Selective breeding on *natural antibodies* in chickens

Tom V. L. Berghof

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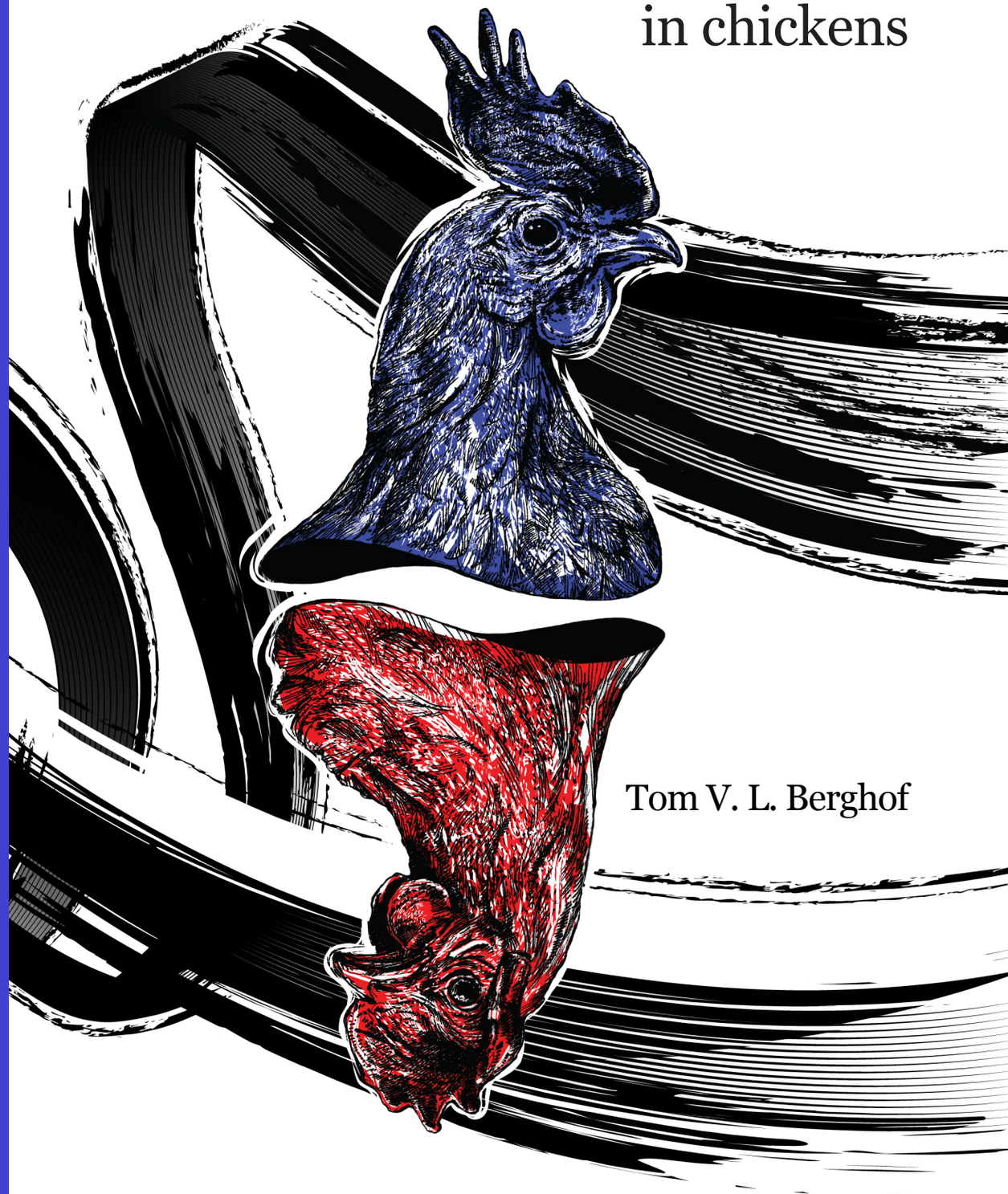
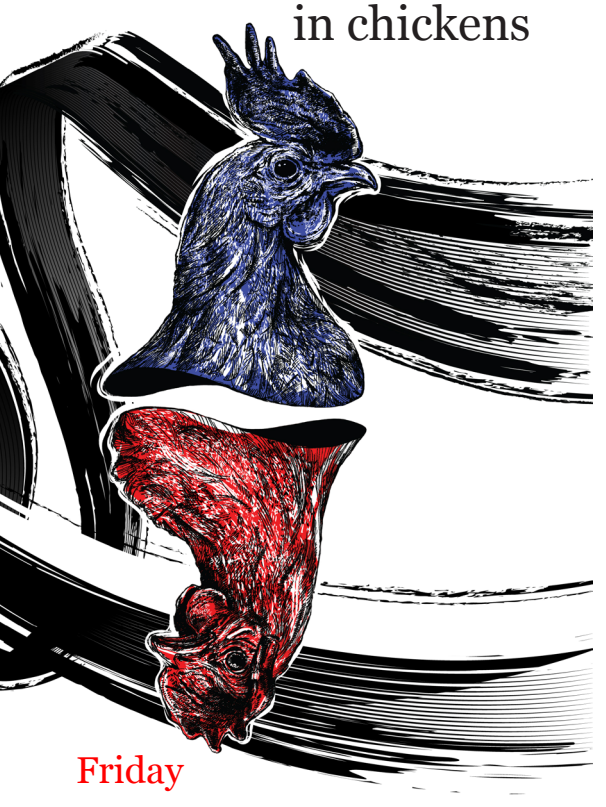


Tom V. L. Berghof

INVITATION

to attend the public defense
of my PhD thesis entitled:

Selective breeding on *natural antibodies* in chickens



Friday
19th of January 2018
at 16:00 hours

At 'Aula' of Wageningen University & Research | Generaal Foulkesweg 1, Wageningen

The defense will be followed by a reception at 'Eetcafé H41'. | Herenstraat 41, Wageningen

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Propositions

1. Selective breeding on higher levels of KLH-binding *natural antibody* titers at 16 weeks of age increases resistance against bacterial diseases in chickens. (this thesis)

2. Toll-like receptors are essential for IgM *natural antibody* levels in chickens. (this thesis)

3. *P*-values are significantly overrated.

4. Societal exposure is critical for scientific development.

5. The term 'Humane endpoint' in animal experiments should be replaced by 'Species-specific endpoint'.

6. The chicken came before the chicken egg.

Propositions belonging to the thesis entitled:
"Selective breeding on *natural antibodies* in chickens"

Tom V. L. Berghof
Wageningen, 19 January 2018

Selective breeding on *natural antibodies* in chickens

Tom V. L. Berghof

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This research was conducted under the auspices of the Graduate School Wageningen Institute of Animal Sciences

Selective breeding on *natural antibodies* in chickens

Tom V. L. Berghof

Thesis
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by the authority of the Rector Magnificus,
Prof. Dr A.P.J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Friday 19 January 2018
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Summary

In modern poultry production, high numbers of birds are housed at high densities. Recent changes in production systems, and management have been implemented with additional challenges: the change from battery cages to free roaming systems, and the reduction in preventive use of antibiotics have increased the risk of diseases, and disease spreading. The costs of diseases in poultry production are considerable, and several disease intervention strategies are currently used, or investigated. One potential strategy is to selectively breed chickens for increased general disease resistance. However, general disease resistance is difficult to define. Instead, selective breeding could be done on one, or several indicator traits, that are related to resistance against multiple pathogens. In addition, indicator traits should be heritable, and easy, and cheap to measure. Natural antibodies (NAb) might be a suitable indicator trait for selective breeding for general disease resistance. NAb are antigen-binding antibodies present in healthy individuals without previous exposure to the recognized antigen. NAb have a diverse range of functional roles: they play a role in maintaining homeostasis/housekeeping, regulation of the immune system, preventing auto-immunity, and increasing disease resistance. Moreover, NAb levels binding keyhole limpet hemocyanin (KLH) around adolescence were previously associated with survival in layer chickens, and were estimated to be heritable. Therefore selective breeding on KLH-binding NAb levels around adolescence might be a promising strategy to increase general disease resistance of layer chickens.

The objectives of this PhD thesis were:

1) to investigate the genetic variation of KLH-binding NAb levels in adolescent layer chickens;
2) to investigate the potential of KLH-binding NAb levels as an indicator trait for general disease resistance by
   a) divergently selective breeding on total KLH-binding NAb titers, and
   b) inoculating these NAb selection lines with avian pathogenic *Escherichia coli* (APEC); and
3) to investigate possible correlated selection responses on the immune system, and on production traits.

1) To investigate the genetic variation of KLH-binding NAb levels in adolescent layer chickens, heritabilities were estimated, and genome-wide association studies (GWAS) were performed.

Heritabilities were estimated in the base population of the selection experiment: 3,689 white purebred laying chickens were phenotyped around 16 weeks of age for total KLH-binding NAb titers, and for the isotypes IgM, IgA, and IgG. Heritabilities were 0.12 for total KLH-binding NAb titers, 0.14 for IgM, 0.10 for IgA, and 0.07 for IgG. This means that KLH-binding NAb are heritable, and selective breeding is possible. In addition, high positive genetic correlations were estimated, which means that selective breeding for one NAb type

(i.e. total KLH-binding NAb titers) will result in similar responses of the other NAb (iso)types.

The GWAS were performed with 57,636 single nucleotide polymorphisms (SNP) for total KLH-binding NAb titers, and for the isotypes IgM, IgA, and IgG on 1,628 white purebred laying chickens of the same line as the base population. One genomic region was significantly associated to KLH-binding IgM NAb titers, and to a lesser extent to total KLH-binding NAb titers. The region showed full dominance, and had a major effect on IgM. This region is located on chromosome 4, and contained two Toll-like receptors (TLR). To further characterize the found association, total antibody concentration, and antibody concentrations of the isotypes IgM, IgA, and IgG were measured as well, and GWAS were performed. One genomic region, the same as identified before, was significantly associated to IgM antibody concentration. Full sequence data of key ancestors of the study population allowed imputation to full genome sequence for further association: 16 candidate genes were identified. SNP located in coding regions of these candidate genes were checked for predicted changes in protein functioning. One SNP, a C/G polymorphism at 69,965,939 base pairs (Gallus_gallus-5.0), received the maximum impact score from two independent prediction tools, which makes this SNP the most likely causal variant. The C-variant had an allele frequency of 0.45, showed a dominant mode of gene action, and was associated with high IgM levels. The G-variant had an allele frequency of 0.55, and was associated with low IgM levels. This SNP is located in *TLR1A*, which suggests a fundamental role of TLR1A on regulation of IgM levels, or B cells, or both.

2) To investigate the potential of KLH-binding NAb levels as an indicator trait for general disease resistance, a) layer chickens (originating from the base population) were divergently selectively bred on total KLH-binding NAb titers, and b) two generations of these NAb selection lines were inoculated with avian pathogenic *Escherichia coli* (APEC).

a) The selection criterion was total KLH-binding NAb titers at 16 weeks of age, and selection was based on own performance (i.e. mass selection). Chickens of the base population were selected to either breed the High NAb selection line (High line), or the Low NAb selection line (Low line). Selective breeding was performed for 6 generations. Each generation consisted of approximately 600 chickens per line. The average genetic differences in KLH-binding NAb titers at 16 weeks of age increased per generation with 0.36 for total NAb titers (selection criterion), 0.40 for IgM, and 0.32 for IgG (based on estimated breeding values (EBV)). Selective breeding on total KLH-binding NAb titers at 16 weeks of age was therefore proven to be possible.

Interestingly, the genetic progression of average titers in the High line reduced for KLH-binding IgM NAb titers from generation 4 to generation 6, but was unaffected in the Low line. This could be the result of the dominance effect of the *TLR1A* variants present: in generation 6, the High line females had a C-variant frequency of 0.64, and a G-variant frequency of 0.36, and the Low line females had a C-variant frequency of 0.08, and a G-variant frequency of 0.92. The amount of additive genetic variance, based on the *TLR1A*

variants present in the NAb selection lines, was lower in the High line compared to the Low line: i.e. genetic improvement for higher KLH-binding IgM NAb levels was more difficult compared to genetic improvement for lower KLH-binding IgM NAb levels. Nevertheless, sufficient variation remains present to continue divergent selective breeding on NAb levels, even after six generations of selection.

b) Selective breeding for KLH-binding NAb levels does not necessarily result in differences in general disease resistance. Therefore the NAb selection lines were inoculated with avian pathogenic *Escherichia coli* (APEC). APEC is an opportunistic pathogen, mostly found in the respiratory tract. APEC causes colibacillosis, and can eventually lead to death. It has several antibiotic resistant mechanisms, and vaccination is not sufficiently protective. APEC is therefore a relevant poultry disease to consider. Generation 4, and generation 6 were intratracheally inoculated with one of three doses of APEC at 8 days of age. Mortality was recorded during 7 days, after which the experiment was ended. The observed mortality for all APEC doses was 2 to 3 times higher in the Low line compared to the High line. In addition, the surviving chickens at 15 days of age of the High line appeared to be less influenced by the infection compared to the surviving chickens of the Low line: morbidity scores of colibacillosis were lower, and body weight, and relative organ weights were higher. However, the exact protective mechanism, that underlies APEC resistance, remains to be identified.

3) To investigate possible correlated selection responses on the immune system, and on production traits, several traits were measured at different ages in several generations.

The NAb selection lines were observed for a diverse set of immunological traits at multiple ages during the selection experiment. The High NAb line had, compared to the Low line, higher KLH-binding NAb levels, and other antigen-binding NAb levels in general, a higher specific antibody response against human serum albumin (HuSA), higher antibody concentration, more peripheral B cells, and thrombocytes (percentages), a higher bursa weight, and a higher spleen weight. No line differences were observed for specific antibody responses against KLH, and avian tuberculin purified protein derivative of *Mycobacterium avium* (PPD), peripheral T cells, γδ T cells, NK cells, and antigen-presenting cells (percentages), and liver weight. This suggests that KLH-binding NAb selection has positive correlated selection responses for most immune traits, and no negative correlated responses (for the measured traits).

Several production traits were observed in relation to (selection on) KLH-binding NAb levels. Correlations between KLH-binding NAb types, and several production traits were estimated based on 2,385 females of the unselected base population. A significant positive genetic correlation was found between KLH-binding IgG NAb titers, and the feed conversion ratio (FCR; consumed feed/egg mass produced) ($r = 0.33$). In addition, several significant, though small phenotypic correlations ($r = |0.15|$) were found between KLH-binding NAb levels, and some production traits. In the NAb selection lines, monthly body weight measurements suggest that the High line has a higher growth curve compared to the Low

line. However, the adult body weight did not differ between the NAb selection lines. In generation 5, selected females (35 weeks of age) were monitored for production for three weeks: the High line had a higher egg weight, and whiter eggshells compared to the Low line. The NAb selection lines were equal for eggshell thickness, eggshell breaking strength, and FCR. In general, production traits did not seem to be (strongly) negatively affected by NAb selection, which gives room for simultaneous improvement of KLH-binding NAb levels, and production traits.

Given available studies in literature, and this PhD thesis, I hypothesize that KLH-binding IgM NAb are a proxy for the humoral adaptive baseline immunity. KLH-binding IgM NAb show the potential of an individual's humoral adaptive immune system. This means that not necessarily KLH-binding NAb, but the IgM concentration, or, more likely, the number of naive, resting, IgM-only B cells present in an individual, may determine general disease resistance.

In conclusion, selective breeding for total KLH-binding NAb levels around adolescence in chickens is feasible, and influences APEC resistance at young age. Given available studies in literature, and this PhD thesis, I hypothesize that selective breeding for KLH-binding NAb levels increases bacterial disease resistance, without detrimental effects on the immune system, and production traits. However, more research (e.g. infection experiments with different pathogens, or field experiments) in the NAb selection lines, and in other chicken lines, or chicken breeds, is required to confirm this, and to investigate increased *general* disease resistance.

CHAPTER 1

General introduction

Abstract

Modern poultry production houses large flocks of birds at high stocking density. In combination with recent changes in production systems and management, the risk of diseases, and disease spreading increased. The impact of diseases on economic costs, and animal welfare are considerable, and therefore strategies to increase disease resistance are required: one potential strategy is to selectively breed chickens for increased general disease resistance. Natural antibodies are antigen-binding antibodies present in healthy individuals without a previous exposure to this antigen. Natural antibody levels binding keyhole limpet hemocyanin were previously associated with survival in layer chickens, and natural antibody levels were estimated to be heritable. Therefore selective breeding on natural antibodies might be a promising strategy to increase general disease resistance.

The objective of this thesis was:

1) to investigate the genetic variation of natural antibody levels binding keyhole limpet hemocyanin in adolescent layer chickens;
2) to investigate the potential of KLH-binding NAb levels as an indicator trait for general disease resistance by
   a) divergently selective breeding on total KLH-binding NAb titers, and
   b) inoculating these NAb selection lines with avian pathogenic *Escherichia coli* (APEC); and
3) to investigate possible correlated selection responses on the immune system, and on production traits.

Impact of diseases in poultry production

Modern poultry production keeps flocks with a high number of birds at high stocking density. In combination with the shift from battery cages to free roaming housing systems in 2012 (EU Directive, 1999), the risk of pathogen spreading increased due to more frequent direct contact between chickens (Atkins et al., 2013; Van Bunnik et al., 2014; Rozins et al., 2016). Around that same time, the (abundant) use of preventive antibiotic treatment in livestock production raised concerns, because several bacterial pathogens acquired resistance against several antibiotic types. This resulted in high impact consequences for the poultry industry: nowadays, a high number of chickens is group housed without preventive use of antibiotics (EU Directive, 1999; Neeteson-Van Nieuwenhoven et al., 2016; De Greeff et al., 2017). This makes the poultry industry more vulnerable to diseases, and their impact.

The costs of diseases in poultry production have been estimated to be 10-20% of the gross production value in developed countries (FAO, 2014), and are likely to be higher in developing countries. These costs consist mainly of direct costs, like treatment costs, and loss of income due to mortality. However, these estimates often underestimate total cost, because they do not include, for example, costs for diseases with zoonotic potential, or costs due to (inter)national trade barriers (see Raney et al. (2009), or Robinson et al. (2011) for more information).

The impact of diseases on economical, societal, and animal level in poultry production is high. The impact of diseases can be reduced by application of intervention strategies to prevent, or control disease.

Disease intervention strategies

The impact of diseases can be reduced by applying (a combination of) disease intervention strategies, targeting either the disease (i.e. pathogen), or the animal. Several strategies are known and applied, related to, for example, management and housing, antimicrobials, feed, microbiota, and vaccinations (Humphrey, 2006; Denagamage et al., 2015; Sahin et al., 2015). With a complex problem, like diseases, complementary application of different strategies is the most effective in tackling the problem: every single strategy has advantages, and disadvantages, that can be back-upped by a complementary strategy.

Selective breeding for increased general disease resistance, i.e. a broad, and a-specific disease resistance (Box 1), could be a potentially interesting, complementary strategy. Selective breeding for increased general disease resistance uses the natural potential of a population to increase general disease resistance, and has as major advantage that it increases resistance of all individuals of subsequent generations. Selective breeding for improved general disease resistance can therefore be considered as an important disease intervention strategy in poultry production (Wijga et al., 2009; Cheng et al., 2013).

Breeding for general disease resistance

Selective breeding for an increased general disease resistance would preferably be done based on challenge experiments to select for, or to verify improvement of disease resistance. For example in aquaculture, challenge experiments for certain diseases are being performed, and outcomes are successfully implemented to selectively breed for (specific) disease resistance (Drangsholt, 2011; Bishop et al., 2014; LaFrentz et al., 2016). Also in chickens, it would be relatively easy to perform these challenge experiments, but this has ethical constraints due to severely affected animal welfare. Also, especially for other livestock (e.g. swine, bovine), such challenge experiments are economically costly, because challenge experiments require large number of animals, need to be performed on animals related to the selection candidate(s), need to be performed with many different pathogen models, and can be difficult to standardize. Therefore, selective breeding should be done on one, or several indicator traits (also known as biomarkers, or health parameters), that are related to resistance against several pathogens. Indicator traits are traits that in themselves are not necessarily relevant, but are (genetically) correlated with the trait of interest (Stear et al., 2001). Indicator traits should be heritable, easy and cheap to measure, and, most importantly, related to general disease resistance.

Numerous studies describe (divergent) selection experiments in chickens over the last half century to investigate general disease resistance with different indicator traits (see also Terčič (2013) for a review). Two well studied examples are the high, and low specific antibody (SpAb) responders to sheep red blood cells (Siegel et al., 1980; Van der Zijpp et al., 1986). Others investigated, for example, SpAb response to *Escherichia coli* vaccination (Leitner et al., 1992), different MHC B-haplotypes (Bacon et al., 1992), IgM, or IgG serum concentration (Okada et al., 1987; Sarker et al., 1999), group longevity (Cheng et al., 2001), cell-mediated immune response (Pinard-Van der Laan, 2002; Sundaresan et al., 2005), phagocytosis (Pinard-Van der Laan, 2002; Li et al., 2008), mannan-binding lectin serum concentration (Juul-Madsen et al., 2007), and pro-inflammatory mediators (Swaggerty et al.,

Box 1: General vs. specific disease resistance

'General disease resistance' refers to a broad, and a-specific disease resistance. 'Specific disease resistance' is used in the context of resistance against one particular (specific) disease. In this thesis, I will generally use the term 'general disease resistance' to stress the broad, and a-specific resistance against several (types of) diseases.

Technically, 'disease resistance' is an incorrect term: resistance refers to "the ability of the host animal to exert control over a parasite, or pathogen lifecycle" (Bishop, 2012), while disease is the negative physiological consequence of an (established) infection (Bishop et al., 2014). Better terms are 'resistance', 'pathogen resistance' (Bishop et al., 2014), or 'susceptibility' (Lipschutz-Powell et al., 2012).

2014). However, except for elimination of certain MHC B-haplotypes (haplotype associated with low disease resistance for some diseases), none have been applied in breeding practices, as far as I know. Reason(s) for this can only be speculated about: the trait was not, or was poorly heritable (i.e. no genetic variation), the trait was too expensive to measure, the trait was too laborious to measure on many individuals, the trait was not indicative for multiple (i.e. general) disease resistance, the trait could not be tested on breeding candidates themselves, or, more likely, a combination of these reasons. In addition, undesirable correlated (selection) responses (Box 2) could have been present on production traits due to increased energetic costs of the immune system, which is explained by various theories (e.g. the 'resource allocation theory' (Rendel, 1963; Beilharz et al., 1993)). Although others suggested that this increase in energetic costs, even during activation, is low (Klasing (1998); Iseri et al. (2013); see also Lee (2006) for a review). Nevertheless, serious negative effects of selection for an indicator trait on production are undesirable, and production should be monitored.

Natural antibodies

Natural antibodies (NAb) might be a suitable indicator trait for selective breeding for general disease resistance. NAb are immunoglobulins present in the healthy organism recognizing an antigen in the absence of previous exposure to this antigen (Vale et al., 2016), unlike SpAb made after immunization. Several studies have associated higher NAb binding keyhole limpet hemocyanin (KLH) at 20 weeks of age with increased survival during the first part of the laying period (up to 60 weeks of age) in layer chickens (Star et al., 2007a; Sun et al., 2011; Wondmeneh et al., 2015). In addition, the majority of studies regarding NAb describe beneficial effects of higher NAb on reduced mortality, and increased disease resistance in several species (Table 1).

NAb are part of the adaptive immunity, and can in that capacity be considered as the potential

Box 2: Correlated selection response

A 'correlated (selection) response' is the change in trait Y as a consequence of selection for trait X. Trait X, and trait Y are genetically correlated, which means these traits are either in linkage disequilibrium (one genomic region containing two, or more genes each influencing trait X, or trait Y), or pleiotropy (one genomic region containing one gene influencing trait X, and trait Y) (Saltz et al., 2017). Pleiotropy is generally considered to be the major cause of correlated responses (Rauw, 2009).

Selection experiments in the past have often encountered positive, and negative correlated response to the selection criterion on, for example, production traits, or immune(-related) traits (e.g. Rauw et al. (1998); Rauw (2009); Van der Most et al. (2011)).

Table 1. A selection of literature reporting on effects of natural antibodies (NAb) in blood (unless stated otherwise) in several animal species. The table shows the type of NAb, the study population, the associated effect of higher NAb levels, and the reference(s).

NAb	Study population	Associated effect	Reference(s)
KLH-binding	Layer hens	Increased survival	Star et al. (2007a), and Sun et al. (2011)
		No effect on survival	Sun et al. (2013b)
		Increased survival	Wondmeneh et al. (2015)
	Indigenous Ethiopian hens	Decreased survival	Wondmeneh et al. (2015)
RRBC-binding (agglutination)	Barn Swallows	No effect on survival	Møller et al. (2007)
KLH-binding	Rainbow trout	Increased resistance to furunculosis	Michel et al. (1990)
A-protein[1]-binding	Goldfishes	Increased resistance to *A. salmonicida* (morbidity)	Sinyakov et al. (2002)
KLH-binding	Soay sheep	Reduced survival, and lower body weight	Nussey et al. (2014)
KLH-binding	Dairy cows	Reduced risk of mastitis	Ploegaert (2010), and Thompson-Crispi et al. (2013)
KLH-binding in milk		Reduced longevity	De Klerk (2016)
IgM	Mice	Several positive effects on controlling bacterial, viral, and parasitical pathogens	Table 1 of review by Ehrenstein et al. (2010)
Several	Humans	Protective role in fungal infections, and inflammation	Review by Elluru et al. (2015)
Auto-reactive		Beneficial control of inflammatory, and autoimmune disease	Review by Grönwall et al. (2014)

[1] **A-layer-protein (A-protein) is the major protein constituent of *Aeromonas salmonicida*.**

of the humoral adaptive immune system, representing the humoral adaptive baseline of the innate immunity (Adelman et al., 2004; Soler et al., 2011). NAb play a role in maintaining homeostasis/housekeeping, regulation of the immune system, preventing auto-immunity, and increasing disease resistance (Boes, 2000; Adelman et al., 2004; Zinkernagel, 2012). NAb work through opsonisation, and thereby facilitate phagocytosis, facilitate complement activation, and initiate the adaptive immune response (Boes (2000); Ehrenstein et al. (2010); Panda et al. (2015); see also Born et al. (2016) for an overview). NAb are characterized as polyreactive antibodies with low affinity, because of little, or no somatic hypermutation (Ochsenbein et al., 2000; Zhou et al., 2007; Wang et al., 2016). NAb have also been found in germfree mice (Born et al., 2016), which suggests that antigen-recognition of NAb is germline encoded instead of environmentally induced somatic hypermutation (Casali et al., 1996; Boes, 2000; Chen et al., 2015). However, NAb have also shown to respond to stimuli (Haghighi et al., 2006; Berghof et al., 2010; Vale et al., 2016), and to be affected by aging (Parmentier et al., 2004a; Toropainen et al., 2005; Sun et al., 2011). NAb bind phylogenetically conserved structures (e.g. damage-associated molecular patterns (DAMP),

and microbe-associated molecular patterns (MAMP)) (Boes, 2000; Ochsenbein et al., 2000; Wang et al., 2016), and therefore can recognize various types of pathogens (Table 1).
Different NAb isotypes are found: predominantly IgM, but also IgA, and IgG (Panda et al., 2013; Baumgarth et al., 2015; Panda et al., 2015). It suggests that class-switching from IgM to IgA, or IgG is relatively limited, or is strictly controlled in NAb-producing B cells (Box 3). Alternatively, natural antibodies might be produced by 'normal' B cells. Interestingly, most of the described effects are only described for, or are strongest for IgM NAb (Table 1).
In summary, NAb levels are, because of their role, and wide range of involvement in the immune system, a possible indicator trait for general disease resistance.

KLH-binding natural antibodies in adolescents as indicator trait

Selective breeding should be based on an indicator trait, which should be heritable, easy and cheap to measure, and, most importantly, related to general disease resistance. Previous studies suggest that KLH-binding NAb in adolescent chickens might be a suitable indicator trait for general disease resistance (Star et al., 2007a; Sun et al., 2011; Wondmeneh et al., 2015) KLH-binding NAb are easy, and relatively cheap to measure, and do not require

Box 3: Natural antibody-producing B cells

Originally, NAb in mice and humans were thought to be solely produced by one subpopulation of B cells: the B1 B cell. However, new insights have included many new NAb-producing B cell types at different locations, and it is now suggested that "**well-defined [NAb]-synthesizing B-cell subsets apparently do not exist, but are rather a continuum of functional and phenotypic B-cells that cannot be clearly defined** (Tumas-Brundage et al., 2001)" (cited from Avrameas (2016)). This fits with the absence of success to define the NAb-producing B cell in livestock species (i.e. swine, sheep, and bovine) this far (Van Altena et al., 2016; Van Altena, 2016). Although some NAb-producing B cell phenotypes are more present at certain locations: the B1 B cell population, of which many reside in the peritoneal, and pleural cavities (Baumgarth, 2011; Savage et al., 2015), and the marginal zone B cell population in the spleen (Durand et al., 2009). B cells of these locations have received little attention as a possible source of NAb in livestock species, including in chickens. As far as I know, no studies have investigated B cells in the peritoneal, or pleural cavities in chickens. In addition, the chicken spleen does not (morphologically) contain B cell follicles, and marginal zones, as in mammals (Ratcliffe et al., 2014), so detection of marginal zone B cells is complicated.
In summary, a distinct NAb-producing B cell population has not been identified (yet?) in chickens, but some possible locations for these cells can be presumed.

(invasive) treatments. KLH is a large protein with many, and many different epitopes (Box 4). KLH can therefore be recognized by relatively many, and many different KLH-binding NAb, and might therefore give a good representation of the total NAb level of an individual. However, selective breeding for KLH-binding NAb levels is only possible if these NAb levels are heritable.

Few studies have investigated heritabilities of NAb in chickens. Only two studies estimate heritabilities of KLH-binding NAb in adolescent chickens: Sun et al. (2013a) estimated KLH-binding NAb heritabilities in female purebred layer chicken lines at 20 weeks of age to be 0.41 for IgM, and to be 0.31 for IgG (Sun et al., 2013a). Sun et al. (2013b) estimated KLH-binding NAb heritabilities of female crossbred layer chickens at 24 weeks of age to be 0.26 for IgM, and to be 0.21 for IgG (Sun et al., 2013b). In both studies, maternal effects were not, or could not be estimated, although possibly present (Sun et al., 2013b), which affect heritability estimates if present. Three other studies also investigated heritabilities of NAb, but binding different antigens (KLH, rabbit red blood cells, or sheep red blood cells (SRBC)) at different ages (4, 5, 40, and 65 weeks of age) in different populations (crossbred, purebred, and SRBC-binding SpAb selection lines): these ranged from approximately 0.03 to 0.44 (Van der Zijpp et al., 1980; Wijga et al., 2009; Sun et al., 2013a). Two studies investigated genomic regions underlying genetic variation of KLH-binding NAb levels at 20 weeks of age by using dedicated single nucleotide polymorphism (SNP) sets with 1,022 SNP (Biscarini et al., 2010; Sun et al., 2013a), but none of the SNP gave a strong association with

Box 4: Keyhole Limpet Hemocyanin

Keyhole limpet hemocyanin (KLH) is the copper-containing extracellular oxygen-carrier protein from the *Megathura crenulata*, a sea snail native to the Pacific coast of California (USA), and Mexico. **It is extremely unlikely that chickens have encountered KLH in their life, and therefore antibodies binding KLH can be considered NAb** (according to the definition of NAb).

KLH contains 7, or 8 functional unit domains, and has a molecular weight of approximately 350 to 400 kDa in total (Harris et al., 1999). KLH received, and still receives, a lot of attention in the field of immunology, because of its T helper 1 (Th1) cell-activating, and Th2 cell-activating properties (Chow et al., 2012). This might be the result of KLH's possible binding to mannose, and fucose C-type lectin receptors on antigen-presenting cells (Sundsmo et al., 2012). *N*-glycans of KLH, and *Schistosoma mansoni* were found to share structural similarities (Geyer et al., 2005; Sundsmo et al., 2012), and more recent work also showed shared similarities between KLH, and *Trypanosoma*, *Leishmania*, *Cryptococcus*, *Aspergillis*, *Candida*, *E. coli*, *Salmonella*, *Clostridium*, and Hepatitis C virus (Sundsmo et al., 2012).

For a review on KLH, see Harris et al. (1999). For more information on shared *N*-glycan structures, see the conference abstract, and conference poster of Sundsmo et al. (2012).

NAb. In summary, previous studies suggest that NAb are heritable, especially IgM, but presence of maternal effects has to be checked (Wijga et al., 2009; Sun et al., 2013b), and little is known about the genes underlying NAb variation.
To conclude: NAb are related to general disease resistance, are heritable, and are easy, and (relatively) cheap to measure, and therefore KLH-binding NAb in adolescents might be a suitable indicator trait.

Objective and outline of this thesis

The objectives of this thesis were:
1) to investigate the genetic variation of natural antibody levels binding keyhole limpet hemocyanin in adolescent layer chickens;
2) to investigate the potential of KLH-binding NAb levels as an indicator trait for general disease resistance by
   a) selective breeding on total KLH-binding NAb titers, and
   b) inoculating these NAb selection lines with avian pathogenic *Escherichia coli* (APEC); and
3) to investigate possible correlated selection responses on the immune system, and on production traits.

Two study populations were used to investigate these objectives: an unselected layer chicken population (referred to as the base population), and a population of several (up to six) generations of layer chickens divergently selectively bred for KLH-binding NAb levels at adolescence originating from the base population (referred to as the NAb selection lines).
Figure 1 gives an overview of the different topics of each chapter. In chapter 2, I investigated heritabilities of KLH-binding NAb levels in the unselected population. In chapter 3, I investigated the correlations between KLH-binding NAb levels, and several production traits in the unselected female population, in order to identify possible unfavorable correlated responses of NAb selection on production traits, and vice versa. In chapter 4, I performed genome-wide association studies (GWAS) to identify genetic regions underlying KLH-binding NAb levels in the unselected population, and I used sequence data to predict the most likely causal variant. In chapter 5, I investigated the correlated response of NAb selection on the humoral adaptive immune system (both NAb, and SpAb) in the selected female population of generation 2 by means of an immunization experiment. In chapter 6, I evaluated the effect of selection on high, and low levels of KLH-binding NAb for avian pathogenic *Escherichia coli* (APEC) resistance at young age in generation 4 and generation 6. In chapter 7 (the 'General Discussion' of my thesis), I reflected on my work, and evaluated if the three objectives of my thesis have been achieved. I also placed my work in a broader context, formulated recommendations for industry, and defined future research areas.

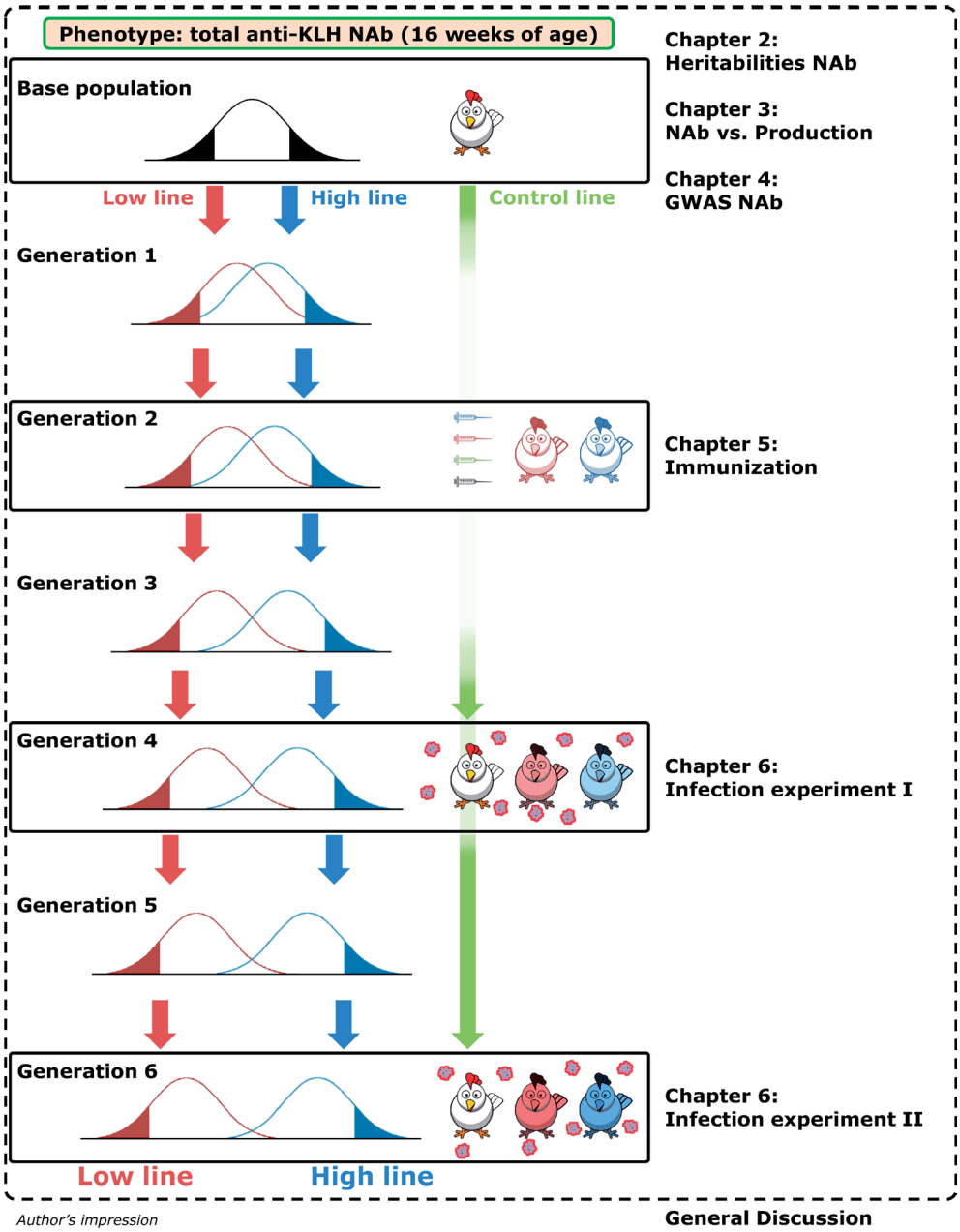

Figure 1. Overview of this thesis' topics per chapter, related to the used study populations. The phenotype of interest is total natural antibody (NAb) levels binding keyhole limpet hemocyanin (KLH) around 16 weeks of age, which is studied in two populations: an unselected, base population (used in chapter 2, chapter 3, and chapter 4), and the NAb selection lines, selected from the base population for total KLH-binding NAb titers at 16 weeks of age (used in chapter 5, and chapter 6).

References

See List of references at the end of this thesis.

Supplementary Information

This thesis is part of the project 'Divergent selection for natural antibodies in poultry' on investigation of KLH-binding NAb as an indicator trait for general disease resistance, granted by "NWO-TTW" (project 12208), and in collaboration with "Hendrix Genetics". The information below was taken from the project proposal.

The research objectives of this project are: 1. to identify genetic variation underlying NAb levels in chickens by performing GWAS; 2. to divergently select chickens for high and low NAb levels; 3. to study consequences of divergent selection on NAb levels on other (production, and health) traits in the selection lines; 4. to characterize candidate genomic regions in depth by using next generation sequencing; and 5. to breed a sixth generation, that will be evaluated for performance and health under recurrent field test conditions in crossbred offspring.

The targets of the research project are: 1. to generate knowledge of relations between NAb (plasma) levels, morbidity, mortality, resistance to infection, vaccine responses, and sustained egg lay of poultry, respectively, using association studies, and specific selection lines; 2. to validate, and identify (new) genomic regions associated with these parameters; 3. to provide the poultry breeding industry a new tool to be implemented in genomic selection, or marker assisted selection breeding strategies for improved health; 4. to increase knowledge of immunology, and mechanisms of (non-specific general) disease resistance, and the genetic relations between immune response genes, and 'production' genes; and 5. to reduce use of medication, and antibiotics, and to obtain healthier birds, which will ultimately result in reduced costs of poultry industry, and might also result in safer products (i.e. eggs, and meat) for consumers.

CHAPTER 2

Genetic and non-genetic inheritance of natural antibodies binding keyhole limpet hemocyanin in a purebred layer chicken line

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with minor changes to Supplementary Information

Abstract

Natural antibodies (NAb) are defined as antibodies present in individuals without known antigenic challenge. Levels of NAb binding keyhole limpet hemocyanin (KLH) in chickens were earlier shown to be heritable, and to be associated with survival. Selective breeding may thus provide a strategy to improve natural disease resistance. We phenotyped 3,689 white purebred laying chickens for KLH binding NAb of different isotypes around 16 weeks of age. Heritabilities of 0.12 for the titers of total antibodies (IgT), 0.14 for IgM, 0.10 for IgA, and 0.07 for IgG were estimated. We also estimated high, positive genetic, and moderate to high, positive phenotypic correlations of IgT, IgM, IgA, and IgG, suggesting that selective breeding for NAb can be done on all antibody isotypes simultaneously. In addition, a relatively substantial non-genetic maternal environmental effect of 0.06 was detected for IgM, which may reflect a transgenerational effect. This suggests that not only the genes of the mother, but also the maternal environment affects the immune system of the offspring. Breaking strength and early eggshell whiteness of the mother's eggs were predictive for IgM levels in the offspring, and partly explained the observed maternal environmental effects. The present results confirm that NAb are heritable, however maternal effects should be taken into account.

Keywords: Chicken, Natural antibody, Heritability, Maternal environmental effect, Transgenerational

Introduction

Natural antibodies (NAb) are defined as antigen binding antibodies present in individuals in the absence of immunization, vaccination, or previous infection with this antigen (Baumgarth et al., 2005). NAb may serve as first line of defense, likely contributing to disease resistance (Zinkernagel, 2012). NAb isotypes found are IgM, IgA, and IgG (Ochsenbein et al., 1999; Boes, 2000). Two NAb types are distinguished: 1) cryptic antibodies directed to self-, and altered self (neo-)antigens, which become visible after cell damage, and 2) overt antibodies directed to non-self antigens (Lutz et al., 2009). Cryptic NAb may perform homeostatic roles like clearance of cell waste, dead or metabolic materials (Reid et al., 1997), and regulation of cytokines (Balsari et al., 1997). Overt NAb likely act as an early defense barrier, preventing infection and facilitating specific immunity (Tomer et al., 1988; Lammers et al., 2004; Zinkernagel, 2012). NAb were previously found and described in fish (Michel et al., 1990), reptiles (Ujvari et al., 2011), domesticated and wild birds (Parmentier et al., 2004a; Pap et al., 2015), and various mammals (Lutz, 2007). In earlier studies, high NAb levels binding the overt antigen keyhole limpet hemocyanin (KLH) were related to lower mortality of layers (Star et al., 2007a; Sun et al., 2011). Levels of NAb binding rabbit red blood cells (RRBC) (Wijga et al., 2009), sheep red blood cells (SRBC) (Van der Zijpp et al., 1980), and KLH (Sun et al., 2013a; Sun et al., 2013b) were shown to be heritable in poultry. Genomic regions underlying NAb levels were also identified before (Biscarini et al., 2010; Sun et al., 2013a).
Modern poultry production is facing high impact changes in production systems and management. Battery cages are banned and substituted by free roaming systems, which may enhance the risk of infections. In addition, preventive use of antibiotics is strongly inadvisable, because of increasing risks for resistance in animal and human diseases. This stresses the importance of alternative ways to maintain or enhance disease resistance in poultry. Genetic selection for a higher general disease resistance might be such a strategy. For this purpose, traits reflecting disease resistance are required. These traits should be heritable, easy to measure, and related to general disease resistance. NAb titers might be a good candidate trait, but little is known of the genetic background of NAb to evaluate the opportunity of selection.
The present study describes genetic parameters of NAb binding KLH in a purebred white leghorn line population of approximately 16 weeks of age, which contained 3,689 chickens with observations for total KLH binding immunoglobulin (IgT), and the isotypes IgM, IgA, and IgG. Heritabilities, maternal effects, and genetic and phenotypic correlations were estimated within this chicken line.

Materials and Methods

Ethics statement

Samples and data were collected according to Institut de Sélection d'Animale (ISA) protocols, under the supervision of ISA employees. Samples and data were collected as part

of routine animal data collection in a commercial breeding program for layer chickens in The Netherlands. Samples and data were collected on a breeding nucleus of ISA for breeding purposes only, and is a non-experimental, agricultural practice, regulated by the Act Animals, and the Royal Decree on Procedures. The Dutch Experiments on Animals Act does not apply to non-experimental, agricultural practices. An ethical review by the Statement Animal Experiment Committee was therefore not required. No extra animal discomfort was caused for sample collection for the purpose of this study.

Study population

The study population was previously described by Van der Klein et al. (2015).
The purebred white leghorn chicken line (in other work referred to as "WA") is a layer chicken line selected mainly for egg production. In addition, egg characteristics are included in the breeding goal.
Plasma of the studied chicken population (n = 3,689) was collected at 15 weeks of age (for males), or 19 weeks of age (for females), without anesthesia/analgesia, and was stored at -20°C until use. No chickens were killed for sample collection.
The studied chicken population originated from 314 dams. Chickens hatched at three subsequent moments with a 2 week interval (dam age: 50 to 60 weeks). Males were group housed with 12 to 14 males until wk 18 of age, and females were grouped house with 15 to 20 females until wk 18 of age. Subsequently all birds were individually housed. Chickens received a standard rearing diet 1 until wk 8, a standard rearing diet 2 from wk 8 until wk 16, and a standard laying diet from wk 16 until the end of the laying period (all commercially available diets). Feed was provided ad libitum, and water was provided ad libitum (wk 0 to 16), or 15.5 h per day (wk 16 until the end of record period). The light regime was 24 h light per day at hatch, and was weekly, gradually reduced to 19 h light per day at 11 weeks of age. The chickens received obligatory vaccinations against Marek's disease (d 1 intramuscular (i.m.)), infectious bronchitis (d 1, d 12-14, wk 10, wk 12 via spray; wk 16 i.m.), Newcastle disease (d 13, d 42, wk 12 via spray; wk 16 i.m.), infectious bursal disease (d 25 via spray; wk 16 i.m.), chicken anemia virus (wk 16 via water), fowl pox (wk 16 by wing web injection), and avian encephalomyelitis (wk 16 by wing web injection).
In addition, several production traits of all dams of the study population were recorded. Body weight of the dams was recorded once between 35 and 40 weeks of age. Egg production was recorded as the summed number of eggs laid between 17 and 24 weeks of age, 25 and 34 weeks of age, or 35 and 56 weeks of age. Egg weight was based on the average weight of the eggs laid on 7 subsequent days between 17 to 34 weeks of age, or 35 to 56 weeks of age. Eggs less than 30 g, and eggs over 90 g were excluded, as well as double-yolk eggs. Egg breaking strength was recorded as the average breaking strength of all eggs (maximum 6 eggs) laid during a 6 day-period between 25 to 28 weeks of age, or 35 to 56 weeks of age. Egg breaking strength was recorded within one week after collection of the last egg. Egg breaking strength was recorded using the Eggshell tester (FUTURA, Lohne, Germany)

according to manufacturer's manual. Early eggshell whiteness was based on the average of the three first eggs laid. Early eggshell whiteness was recorded with a Konica Minolta spectrophotometer, which was recorded as color values on the L*a*b* color space measurement scale. The L* value represents a black (0) to white (100) scale that was used for early eggshell whiteness measurements. Haugh unit was based on the average of three consecutive eggs laid between 17 to 34 weeks of age, or 35 to 56 weeks of age. Eggs were stored for no more than two days. Haugh unit was recorded using the Albumen height gauge (FUTURA, Lohne, Germany) according to manufacturer's manual. Feed conversion ratio (FCR) was recorded between 33 and 43 weeks of age, and calculated as the feed intake (g) divided by the egg mass produced (g) during a measuring period of three consecutive weeks.

Natural antibodies binding KLH

Titers of total immunoglobulins binding KLH (IgT), and the immunoglobulin isotypes IgM, IgA, and IgG binding KLH were determined in individual plasma samples by an indirect two-step ELISA as described by Van der Klein et al. (2015). Briefly, flat-bottomed, 96-well medium binding plates (Greiner Bio-One, Alphen a/d Rijn, The Netherlands) were coated with 2 µg/mL KLH (Sigma-Aldrich, St. Louis, MO, USA) in 100 µL coating buffer (5.3 g/L Na_2CO_3, and 4.2 g/L $NaHCO_3$; pH 9.6), and incubated at 4°C overnight (o/n). After washing with tap water containing 0.05% Tween 20 for 6 s, plates were tapped dry. Plasma samples were 1:10 pre-diluted (for IgT, IgM, and IgG analyses), or were 1:5 pre-diluted (for IgA analysis) with dilution buffer (phosphate buffered saline [PBS; 10.26 g/L $Na_2HPO_4 \cdot H_2O$, 2.36 g/L KH_2PO_4, and 4.50 g/L NaCl; pH 7.2] containing 0.5% normal horse serum, and 0.05% Tween 20). Pre-dilutions were stored at 4°C until use the next day, or were frozen until use. Pre-dilutions were diluted with dilution buffer. Tested plasma dilution were 1:40, 1:160, 1:640, and 1:2,560 for IgT, IgM, and IgG, or 1:10, 1:20, 1:40, and 1:80 for IgA. Duplicate standard positive plasma samples (a pool of approximately half of the males) were stepwise diluted with dilution buffer. The plates were incubated for 1.5 h at 23°C. After washing, plates were incubated with 1:20,000-diluted rabbit-anti-chicken IgG heavy and light chain (IgT) labeled with horse radish peroxidase (Cat# A30-107P, RRID:AB_67386), or 1:20,000-diluted goat-anti-chicken IgM labeled with horse radish peroxidase (Cat# A30-102P, RRID:AB_66857), or 1:7,500-diluted goat-anti-chicken IgA labeled with horse radish peroxidase (Bethyl Laboratories Cat# A30-103P, RRID:AB_66833), or 1:40,000-diluted goat-anti-chicken IgG(Fc) labeled with horse radish peroxidase (Cat# A30-104P, RRID:AB_66843) (all polyclonal antibodies from Bethyl Laboratories, Montgomery, TX, USA; see also www.antibodyregistry.org), and incubated for 1.5 h at 23°C. After washing, binding of the antibodies to KLH was visualized by adding 100 µL substrate buffer (containing reverse osmosis purified water, 10% tetramethylbenzidine buffer [15.0 g/L sodium acetate, and 1.43 g/L ureumperoxide; pH 5.5], and 1% tetramethylbenzidine [8 g/L TMB in DMSO]). After 15 min (for IgT, IgM, and IgG), or 30 min (for IgA), the reaction was stopped with 50 µL of 1.25 M H_2SO_4. Extinctions were

measured with a Multiskan Go (Thermo scientific, Breda, The Netherlands) at 450 nm. Antibody titers were calculated based on log2 values of the dilutions that gave extinction closest to 50% of E_{MAX}, where E_{MAX} represents the mean of the highest extinction of the standard positive plasma samples, thereby partly correcting for plate differences (Van der Klein et al., 2015).

The repeatability of the ELISA test was examined by randomly choosing 400 individuals of the sampled population. 1:10 pre-dilutions were made twice on two separate occasions. IgT was measured as described above in both sets of samples simultaneously using the same reagents. The Pearson correlation between the two sets was calculated using SAS 9.3.

Statistical analyses

The following linear animal model was used for estimating variance components for IgT, IgM, IgA, and IgG titers binding KLH:

$$y_{ij} = \mu + P_i + a_j + e_{ij} \quad \{1\}$$

where y_{ij} is the IgT, IgM, IgA, or IgG titer, μ is the overall mean, P_i is the fixed effect of plate on which a sample was analyzed (i = 1-188 for IgT, IgM, and IgG, or i = 1-198 for IgA), a_j is the random additive genetic effect of the j[th] animal assumed to be ~N(0, $\mathbf{A}\sigma_a^2$), and e_{ij} is the residual term assumed to be ~N(0, $\mathbf{I}\sigma_e^2$). Assumed (co)variance structures of the random model terms are $\mathbf{A}\sigma_a^2$, and $\mathbf{I}\sigma_e^2$, in which **A** is the additive genetic relationship matrix, σ_a^2 is the additive genetic variance, **I** is an identity matrix, and σ_e^2 is the residual variance. The pedigree used to construct **A** consisted of 4,586 individuals, and was based on 7 generations of ancestors. Note that the plate effect corrects also for other (confounded) effects on the samples, such as sex, storage, and analyses effects.

Heritabilities were calculated as

$$h^2 = \frac{\sigma_a^2}{\sigma_p^2}$$

and phenotypic variance was $\sigma_p^2 = \sigma_a^2 + \sigma_e^2$, where σ_a^2 is the additive genetic variance, and σ_e^2 is the residual variance. The likelihood ratio test was used to test whether estimated heritabilities were significant different from 0, comparing univariate model {1} to an univariate model in which the additive genetic variance was fixed at a small value of 0.0001. The likelihood ratio test was -2ln(Λ[x]), with

$$\Lambda[x] = \frac{\max\,[\,L_0|x]}{\max\,[\,L_1|x]}$$

where L_0 is the likelihood under the null hypothesis with the additive genetic variance fixed at 0.0001, L_1 is the likelihood under the alternative hypothesis without variance components constrained, and x is the given data set. Significance was assessed assuming that the likelihood ratio follows a χ_1^2 distribution.

We compared univariate model {1} without a maternal effect, with an alternative model including a maternal effect {2}:

$$y_{ijk} = \mu + P_i + a_j + d_k + e_{ijk} \quad \{2\}$$

with d_k, a random effect of the k[th] dam, where effects of d_k are assumed to be ~N(0, $\mathbf{I}\sigma_m^2$) with **I** as an identity matrix, and σ_m^2 as the maternal variance. The likelihood ratio test was used to test for significance of maternal environmental effects, comparing univariate model {1} without maternal effects with univariate model with maternal effects {2}. When maternal effects were significant, the heritability, and the contribution of the maternal effect was calculated as

$$h^2 = \frac{\sigma_a^2}{\sigma_p^2} \text{ and } \qquad m^2 = \frac{\sigma_m^2}{\sigma_p^2}$$

and phenotypic variance was $\sigma_p^2 = \sigma_a^2 + \sigma_m^2 + \sigma_e^2$, where σ_a^2 is the additive genetic variance, σ_m^2 is the maternal variance, and σ_e^2 is the residual variance. When maternal effects were significant, the likelihood ratio test was used to test whether the newly estimated heritabilities were significantly different from 0, comparing univariate model {2} to an univariate model in which the additive genetic variance was fixed at a small value of 0.0001. Phenotypic and genetic correlations between NAb titers were estimated based on bivariate analyses using model {1}, or model {2} in case of a significant maternal effect.

To verify whether the measured traits differ between males and females, titers of males and females were treated as different traits. The likelihood ratio test was used to test whether genetic correlations were different from 1. This was done by comparing bivariate models in which all (co)variances were not fixed (except the covariance between plate in both models, which is 0) with the same bivariate model in which all (co)variances were fixed to the previously estimated (co)variances, and the covariance between the additive genetic effects was fixed so that the genetic correlation equals 1. Also heritabilities and maternal effects per sex were estimated with the previously described univariate model {1}, or model {2} in case of a significant maternal effect.

To get insight in the biological grounds of maternal effects on the offspring, production traits of the dams were added separately as fixed effects to model {2}, when maternal effects were significant. Fixed effects were tested for significance by an incremental Wald F statistics analysis. The maximum explained titer difference of significant dam production traits on NAb titers were estimated by

$$titer\ difference_{max} = (trait_{max} - trait_{min}) * R$$

where $trait_{max}$ is the highest observation of a dam's production trait and $trait_{min}$ is the lowest observation of a dam's production trait, and R is the regression coefficient between a dam's production trait, and the titer of the tested NAb type of the offspring.

All statistical analyses were performed using ASReml 4.0 (Gilmour et al., 2014).

Results

Descriptive statistics and heritabilities are shown in Table 1. In total 3,689 chickens, of which 1,297 males, and 2,392 females, were phenotyped for total NAb immunoglobulin (IgT) titers binding KLH around 16 weeks, and isotypes IgM, and IgG titers. Of this population 3,547

chickens, of which 1,258 males, and 2,289 females, were phenotyped for IgA, because some plasma quantities were too low. Mean titers were 7.3 for IgT, 7.5 for IgM, 6.5 for IgA, and 6.3 for IgG titers. Titers (5^{th} percentile to 95^{th} percentile) ranged from 4.9 to 9.6 for IgT, 5.4 to 9.7 for IgM, 4.4 to 8.7 for IgA, and 3.7 to 9.0 for IgG (Table 1). Heritabilities of IgT, IgA, and IgG NAb were estimated with model {1}. For IgM, significant maternal effects were detected, and therefore the heritability of IgM NAb was estimated using model {2}. The heritabilities of NAb binding KLH were estimated to be 0.12 for IgT, 0.14 for IgM, 0.10 for IgA, and 0.07 for IgG. The maternal environmental effects were estimated to be 0.02 for IgT ($p = 0.08$), 0.06 for IgM ($p = 0.002$), 0.01 for IgA ($p = 0.48$), and 0.01 for IgG ($p = 0.30$). Maternal genetic effects were not found to be significant (data not shown). Paternal (environmental and genetic) effects, which could indicate possible transgenerational epigenetic effects, were also not found to be significant (data not shown). The repeatability of the ELISA test was 0.88.

Average NAb titers for males and females were respectively 7.1 and 7.3 for IgT, 6.8 and 7.9 for IgM, 6.4 and 6.6 for IgA, and 5.2 and 6.9 for IgG (Table 2). Although a sex effect is likely present, it was not separately taken into model {1} and {2}, because the sex effect is confounded with the plate effect. However when reanalyzing IgT in the randomly chosen repeatability set, a significant fixed effect of sex, but no significant fixed effect of plate were found. This suggests that male and female significantly differ in their IgT titers, and that analysis plate effects, other analysis effects, storage effects, or batch effects are not so much of influence on the measured IgT titers. When estimating the genetic correlation between males and females, a genetic correlation of 0.98 for IgT was found, which was not significantly different from 1. Also for IgM (0.92), IgA (0.72), and for IgG (0.85) the genetic correlation between the sexes was not significantly different from 1, but IgA almost significantly differed between sex ($p = 0.06$). Heritabilities between sex were (male vs. female): 0.14 vs. 0.09 for IgT, 0.17 vs. 0.15 for IgM (accounting for significant maternal environmental effects in females only), 0.15 vs. 0.11 for IgA, and 0.11 vs. 0.05 for IgG (Table 2).

Table 1. Descriptive statistics, and genetic parameters (phenotypic variance σ_P^2, heritability h^2, and maternal environmental effect m^2) of total KLH binding natural antibody (IgT) titers, and KLH binding IgM, IgA, and IgG isotype titers in a WA leghorn chicken line at 16 weeks of age.

	IgT[a]	IgM[a]	IgA[b]	IgG[a]
Mean (SD)	7.3 (1.4)	7.5 (1.3)	6.5 (1.3)	6.3 (1.6)
Range[c]	4.9 - 9.6	5.4 - 9.7	4.4 - 8.7	3.7 - 9.0
σ_P^2 (SE)[d]	1.86 (0.05)	1.27 (0.04)	1.50 (0.04)	1.68 (0.04)
h^2 (SE)	0.12 (0.03)	0.14 (0.05)	0.10 (0.02)	0.07 (0.02)
m^2 (SE)	NS	0.06 (0.02)[e]	NS	NS

[a] n = 3,689, of which 1,297 males, and 2,392 females

[b] n = 3,547, of which 1,258 males, and 2,289 females

[c] Range shows 5^{th} quintile and 95^{th} quintile, respectively.

[d] σ_P^2 is the phenotypic variance after adjusting for systematic environmental factors accounted for in the model.

[e] Significant maternal environmental effect ($p = 0.002$)

Table 2. Descriptive statistics, and genetic parameters (phenotypic variance σ_p^2, heritability h^2, and maternal environmental effect m^2) of total KLH binding natural antibody (IgT) titers, and KLH binding IgM, IgA, and IgG isotype titers in males and females of a WA leghorn chicken line at 16 weeks of age.

	Males				Females			
	IgT (n = 1,297)	IgM (n = 1,297)	IgA (n = 1,258)	IgG (n = 1,297)	IgT (n = 2,392)	IgM (n = 2,392)	IgA (n = 2,289)	IgG (n = 2,392)
Mean (SD)	7.1 (1.5)	6.8 (1.4)	6.4 (1.4)	5.2 (1.3)	7.3 (1.3)	7.9 (1.1)	6.6 (1.3)	6.9 (1.4)
Range[a]	4.7 - 9.6	4.7 - 9.2	4.1 - 8.8	3.2 - 7.4	5.4 - 9.5	6.1 - 9.7	4.6 - 8.6	5.0 - 9.3
σ_p^2 (SE)[b]	2.22 (0.09)	1.75 (0.07)	1.71 (0.07)	1.71 (0.07)	1.66 (0.05)	1.00 (0.03)	1.38 (0.04)	1.66 (0.05)
h^2 (SE)	0.14 (0.05)	0.17 (0.05)	0.15 (0.05)	0.11 (0.05)	0.09 (0.03)	0.15 (0.06)	0.11 (0.03)	0.05 (0.02)
m^2 (SE)	NS	NS	NS	NS	NS	0.06 (0.02)[c]	NS	NS

[a] Range shows 5th quintile and 95th quintile, respectively.
[b] σ_p^2 is the phenotypic variance after adjusting for systematic environmental factors accounted for in the model.
[c] Significant maternal environmental effect (p = 0.009)

Descriptive statistics of dam production traits are shown in Table 3. Only dam early eggshell whiteness (p = 0.004), and dam egg breaking strength (at 35 - 56 weeks of age) (p = 0.04) were found to significantly influence IgM. Dam early eggshell whiteness was found to explain at maximum 0.42 titer point of IgM, and dam egg breaking strength was found to explain at maximum -0.40 titer point of IgM. However, when the significance threshold value (α = 0.05) is corrected for multiple testing, only early eggshell whiteness remains significant. All other production traits were not found to be significantly associated with IgM titers. IgT, IgA, and IgG titers were not tested, because no significant maternal effect was found (see before).

Genetic and phenotypic correlations are shown in Table 4. Note that when estimating genetic and phenotypic correlations, maternal environmental effects were accounted for IgM. The IgT NAb titer reflects (but is not the sum of) a combination of IgM, IgA, and IgG. This is also observed in very strong genetic (0.97), and moderate phenotypic correlations (0.55) between IgT and IgM, very strong genetic (0.92), and weak phenotypic correlations (0.30) between IgT and IgA, and very strong genetic (0.96), and phenotypic correlations (0.81) between IgT and IgG. Although the genetic correlations between isotypes were very strong (IgM and IgA: 0.81, IgM and IgG: 0.86, IgA and IgG: 0.87), the phenotypic correlations were weak, but positive (IgM and IgA: 0.33, IgM and IgG: 0.26, IgA and IgG: 0.22).

Discussion

Selection for natural antibodies in chicken may be a promising strategy to enhance general disease resistance. NAb are involved in preventing infection (Tomer et al., 1988; Zinkernagel, 2012), and are associated with decreased mortality in

Table 3. Number of dams (n), mean (with SD), and minimum and maximum observation (Min - Max) of the production traits of the dams of the studied population of WA leghorn chickens. The p value indicates significance of the production trait when added as a fixed effects in the model of KLH binding IgM natural antibody titers at 16 weeks of age (not corrected for multiple testing). In case of significance, the estimated regression coefficient (R) is given.

Trait	n	Mean (SD)	Min - Max	p value	R
Body weight (kg)[a]					
wk 35 - 40	313	1.61 (0.111)	1.28 - 2.01	0.79	
Egg production (#)[b]					
wk 17 - 24	314	19 (8.8)	0 - 40	0.49	
wk 25 - 34	314	70 (1.5)	56 - 71	0.80	
wk 35 - 56	313	152 (2.4)	133 - 155	0.96	
Egg weight (g)[c]					
wk 17 - 34	313	53.7 (2.52)	47.3 - 60.8	0.80	
wk 35 - 56	313	58.7 (2.82)	52.4 - 69.7	0.54	
Egg breaking strength (kg)[d]					
wk 25 - 28	314	4.7 (4.05)	3.7 - 5.7	0.84	
wk 35 - 56	262	4.3 (0.44)	3.0 - 5.7	0.04	-0.1543
Early eggshell whiteness[e]	306	88 (1.7)	83 - 92	0.004	0.0471
Haugh unit[f]					
wk 17 - 34	264	866.9 (44.04)	739.0 - 996.0	0.15	
wk 35 - 56	313	829.1 (44.38)	636.0 - 962.0	0.60	
Feed conversion ratio[g]					
wk 33 - 43	260	1.88 (0.148)	1.54 - 2.38	0.83	

[a] Body weight of the dams was recorded once in the indicated period.

[b] Egg production was recorded as the summed number of eggs laid in the indicated period.

[c] Egg weight was based on the average weight of the eggs laid on 7 subsequent days in the indicated period. Eggs less than 30 g, and eggs over 90 g were excluded, as well as double-yolk eggs.

[d] Egg breaking strength was recorded as the average breaking strength of all eggs laid during a 6 day-period in the indicated period. Egg breaking strength was recorded within one week after collection of the last egg.

[e] Early eggshell whiteness was based on the average of the three first eggs laid. Early eggshell whiteness was recorded as color values on the L*a*b* color space measurement scale. The L* value represents a black (0) to white (100) scale that was used for early eggshell whiteness measurements.

[f] Haugh unit was based on the average of three consecutive eggs laid in the indicated period. Eggs were stored for no more than two days.

[g] Feed conversion ratio was calculated as the feed intake (g) divided by the egg mass produced (g) during a measuring period of three consecutive weeks.

Table 4. Estimated genetic correlations (below the diagonal) and phenotypic correlations (above the diagonal) of total KLH binding natural antibody (IgT) titers, and KLH binding IgM, IgA, and IgG isotype titers in a WA leghorn chicken line at 16 weeks of age. SE are shown in parentheses.

	IgT[a]	IgM[a]	IgA[b]	IgG[a]
IgT	-	0.55 (0.01)	0.30 (0.02)	0.81 (0.01)
IgM	0.97 (0.03)	-	0.33 (0.02)	0.26 (0.02)
IgA	0.92 (0.07)	0.81 (0.09)	-	0.22 (0.02)
IgG	0.96 (0.03)	0.86 (0.09)	0.87 (0.10)	-

[a] n = 3,689, of which 1,297 males, and 2,392 females

[b] n = 3,547, of which 1,258 males, and 2,289 females

layers (Star et al., 2007a; Sun et al., 2011). NAb were related with, and also facilitated specific antibody response levels in poultry (Lammers et al., 2004; Cotter et al., 2005). NAb were previously found to be heritable in laying hens (Van der Zijpp et al., 1980; Wijga et al., 2009; Sun et al., 2013a; Sun et al., 2013b), and dairy cows (De Klerk et al.; Thompson-Crispi et al., 2013; Wijga et al., 2013) (S1 Table). SNP association studies found associations of KLH binding NAb with various immune related genes, such as interleukins, MHC, and various chemokine(-receptors) (Biscarini et al., 2010; Sun et al., 2013a). This suggests an immune regulating role of NAb, and a relation with regulatory networks influencing regulatory T cells, and immune balance.

In the present study, the estimated heritability for KLH binding total NAb immunoglobulin (IgT) titers was low, but significantly different from 0. Wijga et al. (2009) reported a heritability for RRBC binding IgT NAb of 0.23. In addition, they reported a relative high phenotypic variance compared to this study. Difference in heritabilities in the current study and the heritability reported by Wijga et al. (2009) might rest on various reasons. First, there are differences between the used populations: a White Leghorn population (present study) versus a Rhode Island Red population (Wijga et al., 2009). White Leghorns have in general less genetic diversity than Rhode Island Reds (Megens et al., 2009). Second, the reported NAb traits differ: titers of NAb binding RRBC at 32 days of age were determined by agglutination (Wijga et al., 2009), which is likely different from KLH binding NAb titers around 16 weeks of age determined by ELISA. Albeit both tests may give comparable results (Özdemir et al., 2011), the age of the bird does influence NAb titers (Parmentier et al., 2004a; Star et al., 2007a; Sun et al., 2011), and thus possibly the genetic parameters. Third, the chicken population examined by Wijga et al. (2009) was selected for more than 20 generations for sheep red blood cells (SRBC) specific antibodies, which are positively correlated (0.15) to RRBC NAb. Although epitope binding of SRBC is likely not similar to epitope binding of RRBC (Wijga et al., 2009). Sun et al. (2013b), and Sun et al. (2013a) estimated higher IgM, and IgG KLH binding NAb heritabilities (0.26 - 0.41 and 0.21 - 0.31, respectively) than estimated in the present study (0.14, and 0.07, respectively). As was indicated by Sun et al. (2013b), their data did not allow accounting for maternal effects, because of too few offspring per dam (Sun et al., 2013a; Sun et al., 2013b). However, estimates of heritabilities based on maternal family relations (dam model) were higher than estimates based on an animal model, indicating possible maternal effects (Sun et al., 2013b). When in the present study maternal environmental effects were not taken into account for IgM, the heritability was 0.29, which is comparable to the IgM heritability found by Sun et al. (2013b). Therefore the heritability reported by Sun et al. (2013b) is likely overestimated. In the present study, the heritability of IgG binding KLH was not influenced by maternal environmental effects, and is relatively low compared to Sun et al. (2013b), and Sun et al. (2013a). The genetic different chicken populations studied (crossbred or combination of several leghorn lines), the hen-only-populations, the different sampling moments (20 or 24 weeks of age), and the maternal (environmental) effects (Sun et al., 2013a; Sun et al., 2013b)

may account for the observed differences in heritability between the earlier studies and the present study.

As far as we know, this is the first study that estimates systemic IgA NAb heritability. The exact function of systemic IgA is unknown, although recent work suggests that systemic IgA plays a role in clearance of invading (intestinal) bacteria (Cerutti, 2008). It can be hypothesized that systemic IgA may reflect mucosal health/immunity, e.g. mucosal IgA, since chicken secrete a substantial part of the mucosal IgA via the blood to the liver, and subsequently to the bile fluid (Lebacq-Verheyden et al., 1974; Rombout et al., 1992). However, it was also demonstrated that serum IgA has different immunological properties (e.g. pro-inflammatory) compared to mucosal IgA (e.g. anti-inflammatory) in human (Van Egmond et al., 2001). As far as we know, no literature elucidates the relation between systemic and mucosal IgA.

As mentioned, IgT is a combination of IgM, IgA, and IgG, which is also reflected in the estimated correlations. However, the relative contribution of IgM, IgA, and IgG to IgT is difficult to quantify. Naive B cells produce IgM, while KLH binding IgA, or IgG B cells might be the result of environmentally induced, non-antigen specific isotype switching of KLH binding IgM B cells, possibly induced via Toll-like receptors. IgM may therefore be more influenced by genetics, while IgA, and IgG may reflect immunomodulating environmental influences (f.e. vaccination), because immunization was shown to increase IgG NAb levels (Berghof et al., 2010), and likely increases IgA NAb levels as well. This is also suggested by the high genetic correlation (suggesting same genetic base level), and the lower phenotypic correlation (suggesting different environmental influences or different responsiveness to environmental influences) between IgM and IgA, and IgM and IgG. This was observed as well in the significant sex effect in the repeatability test, suggesting that phenotypic NAb titers are different between males and females, possibly because of the different number of vaccinations. But the genetic correlations for IgT, IgM, IgA, and IgG between males and females did not significantly differ from 1. This means that these traits are genetically equal between sexes, even though a phenotypically difference between sexes might be present. Nevertheless the strength of the phenotypic correlations with IgT titers suggests that IgT is mainly influenced by IgG (0.81), and to a lesser extent by IgM (0.55), and IgA (0.30).

Compared to the estimated heritability, a relatively high maternal environmental effect on IgM NAb titers was found (6% of the phenotypic variance). No significant maternal effects were found on IgT, IgA, or IgG NAb titers, although reported previously (Wijga et al., 2009). This transgenerational effect on IgM titers in offspring may rest on the transfer of maternal antibodies via the egg (Berghof et al., 2013). IgM, and IgA antibodies are mainly present in the albumen of the egg, while IgG antibodies are mainly present in the egg yolk. A maternal effect on IgA could be expected if maternal antibodies (in the albumen) are the true carriers of the effect, but this effect was absent. In addition, when examining males and females separately, only female IgM NAb titers were found to be larger, and to be significantly affected by maternal environmental effects ($m^2 = 0.06$, $p = 0.009$) compared to males

(m^2 = 0.03, p = 0.30). This could suggest that: 1) maternal IgM antibodies are not a major contributor to the maternal environmental effects, 2) female chickens are more prone to maternal environmental effects than male chickens, and 3) IgM NAb influencing genes are located on the W chromosome (Sun et al., 2013a). However, splitting up the data compromises the power to detect significant maternal effects, since number are reduced in both populations (especially in the male population). In addition, the genetic correlations for IgM between males and females did not significantly differ from 1, meaning that the traits are the same between sexes. It seems therefore unlikely that maternal environmental effects on IgM in males are absent.

To further analyze the factors that contribute to the observed maternal environmental effects, additional data of all dams of the offspring were examined. When adding these production data separately as fixed effects to model {2}, the average eggshell whiteness of the first 3 laid eggs by the dam, and the average breaking strength of 6 consecutive eggs laid by the dam between 35 and 56 weeks of age were found to be significant. It should be noted that when a correction for multiple testing is applied, only early eggshell whiteness remains significant, but not breaking strength. Early eggshell whiteness was found to explain at maximum 0.42 titer point of IgM, meaning that dams with high IgM levels produced whiter eggshells. Breaking strength was found to explain at maximum -0.40 titer point of IgM, meaning that dams with high IgM levels produced weaker eggshells in the measured period. When both effects were analyzed in one model, only early eggshell whiteness was found to be significant (p = 0.03), and egg breaking strength tended to be significant (p = 0.11). Both effects were only found to be significant when examining the whole population, but were absent or less pronounced when examined on males or females only (data not shown). This could be due to compromised power to detect the effects, because of the reduced population sizes. These production traits were not found to phenotypically or genetically correlate to IgM within the studied population of female chicken (Van der Klein et al., 2015). Due to an insufficient number of dams, we were not able to calculate genetic correlations between breaking strength, and early eggshell whiteness. However, within the described population, the phenotypic correlation was estimated to be -0.10, and the genetic correlations was estimated to be -0.25 (data not shown), meaning whiter eggs have a weaker eggshell, e.g. lower breaking strength. This relation has not been reported before in literature, but correlations ranging between -0.13 and -0.21 were observed in other ISA chicken populations (J. Visscher, ISA; personal communication). A possible explanation might be found in the protection of the offspring: offspring can be protected by increasing the breaking strength of the egg, and by increasing IgM titers in the offspring (possibly via maternal antibodies) with a correlated response of early eggshell whiteness. Increased breaking strength protects the egg against physical forces on the egg, and protects against pathogenic penetration of the egg. Maternal antibodies protect the egg from infection, and also protects the offspring in early life. The correlated response between early eggshell whiteness, and IgM titers in the offspring might be the result of differences in maturation of the dams by estrogen (Zheng et al., 1997), or might be the result of the time the egg spent in (certain parts of) the oviduct,

e.g. shell gland. Future analyses of egg data should give more insight in the relation between early eggshell whiteness, and breaking strength, and whether maternal antibodies in the egg are correlated to these. Other production parameters of the dams, e.g. egg production, egg weight, body weight, feed conversion ratio, and Haugh unit were not significantly contributing to the maternal environmental effect on IgM.

In summary, this study shows that levels of natural antibodies (IgT, IgM, IgA, and IgG) binding KLH are heritable, and provide a strategy to selectively breed for improved natural disease resistance in poultry. However, it remains to be studied whether selection for NAb might cause an (indirect) correlated response for production traits. In chickens divergently selected for primary specific antibody responses, it was found that high responders had lower body weight, decreased egg production, decreased egg weight, and later first egg production compared to low responders (Adriaansen-Tennekes et al., 2009; Parmentier et al., 2012). The correlated responses of NAb titers, and production parameters in the described layer chicken population are discussed elsewhere (Van der Klein et al., 2015). Finally, studies are in progress to determine the genetic background (SNP association studies) of NAb levels binding KLH, and whether divergently breeding for levels of NAb levels binding KLH also enhances or decrease immune responses to other antigens.

Conclusion

The present study confirms that levels of NAb binding KLH titers around 16 weeks of age in a purebred line are heritable. Also we show that selection for total levels of immunoglobulins (IgT) binding KLH are highly correlated to IgM, IgA, and IgG isotypes binding KLH, making it possible to influence all isotypes simultaneously in the same direction by selecting for high or low NAb. However, IgM titers were relatively strongly influenced by maternal environmental effects, which should be taken into account for future studies.

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Literature cited

See List of references at the end of this thesis.

S1 Table *(adjusted)*. Literature overview of heritabilities of natural antibodies. Literature overview of estimated heritabilities, and maternal effects (m^2) of total natural antibody (IgT) titers, and IgM, IgA, and IgG isotype titers binding different antigens. Hyphen (-) means the parameter was not estimated in that study.

Specie	Population	Sex	Antigen[a]	Age	IgT	IgM	IgA	IgG	m^2 [b]	Reference(s)
Chicken	Crossbred	Both	RRBC	4 wk	0.23	-	-	-	0.10[c]	Wijga et al. (2009)
	Crossbred	Both	SRBC	5 wk	0.43 - 0.03[d]	-	-	-	-	Van der Zijpp et al. (1980)
	Crossbred	Female	KLH	24 wk	-	0.26	-	0.21	-	Sun et al. (2013b)
	Purebred lines	Female	KLH	20 wk	-	0.41	-	0.31	-	Sun et al. (2013a)
				40 wk	-	0.42	-	0.14	-	Sun et al. (2013a)
				65 wk	-	0.44	-	0.26	-	Sun et al. (2013a)
Cow	Dutch Holstein	Female	KLH	4.3 - 6.0 yr	-	0.25	-	0.25	-	De Klerk et al.
	Canadian Holstein	Female	KLH	Unknown	-	0.18	-	0.32	-	Thompson-Crispi et al. (2013)
	Dutch Holstein	Female	KLH	Unknown	0.40 - 0.34[e]	0.55 - 0.48[e]	0.40 - 0.32[e]	0.45 - 0.41[e]	-	Wijga et al. (2013)

[a] KLH: keyhole limpet hemocyanin; RRBC: rabbit red blood cells; SRBC: sheep red blood cells

[b] When maternal (environmental and genetic) effects were not estimated, direct heritabilities can be overestimated when maternal effects are actually present.

[c] Maternal environmental effect

[d] Heritabilities were estimated on sire variance or dam variance, respectively. Note: estimated heritability was read out from figure.

[e] Heritabilities were estimated on intraherd or across-herd variance, respectively.

CHAPTER 3

Genetic relations between natural antibodies binding keyhole limpet hemocyanin and production traits in a purebred layer chicken line

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Abstract

Natural antibodies (NAb) are an important component of the first line of immune defense. Selective breeding for enhanced NAb levels in chickens may improve general disease resistance. It is unknown what the consequences of selection for NAb will be on the productive performance of laying hens. In this paper we describe the genetic relations between NAb titers binding keyhole limpet hemocyanin (KLH) at 19 weeks of age and production traits in a white purebred leghorn chicken line observed in several time periods. A linear animal model was used to estimate (co)variance components, heritabilities, and correlations. Negative genetic correlations were found between egg weight and NAb titers, and between egg breaking strength and NAb titers. Positive genetic correlations were found between the feed conversion ratio (consumed feed/egg mass produced) and NAb titers, and egg production and NAb titers. Negative phenotypic correlations were found between body weight and NAb titers, between egg weight and NAb titers, and between egg breaking strength and NAb titers. Positive phenotypic correlations were found between egg production and NAb titers, and feed conversion ratio and NAb titers. In general, phenotypic correlations were more often significant, but less pronounced than genetic correlations. Other production traits were not found to be significant related to NAb titers. These findings suggest that there is a genetic trade-off between levels of immunity and some production traits, although the underlying mechanism(s) remain(s) unclear. The results suggest possible consequences for production efficiency as a result of selective breeding for improved general disease resistance by natural antibodies.

Keywords: Poultry, Natural antibody, Production, Genetic, Resource allocation

Introduction

In free range poultry housing systems, high stocking densities and close contact between chickens likely increase the risks of infectious diseases. Preventing or treating these diseases is becoming more difficult, due to stringent legislations and limitations set on the use of antibiotics. There is an increased need for a more robust laying hen, i.e. a chicken that maintains egg production and health under varying housing and management environments (Star et al., 2007a). Selective breeding for an improved immune system might be an important tool to improve general disease resistance and in this way reduce the incidence of infectious diseases (Wijga et al., 2009).

Studies with wild birds showed that increased (re)productive effort reduces specific immune responses, parasite resistance, and parent survival (Dijkstra et al., 1990; Deerenberg et al., 1997; Nordling et al., 1998). A trade-off between production and the immune system was also suggested by divergent selection experiments for specific antibody (SpAb) responses in chickens. Selection for increased SpAb responses to sheep red blood cells (SRBC) resulted in lower body weight (Siegel et al., 1980; Parmentier et al., 1996), later maturation (Siegel et al., 1982; Martin et al., 1990; Pinard - Van der Laan et al., 1998), and lowered egg weight in high antibody producing birds (Adriaansen-Tennekes et al., 2009; Parmentier et al., 2012). Selection for an adaptive immune response (e.g. SpAb) may not necessarily result in enhanced general disease resistance, but only in resistance for specific diseases and correlated responses (Kreukniet et al., 1992).

Previously, high levels of natural antibodies (NAb) binding keyhole limpet hemocyanin (KLH) were shown to be associated with survival in both purebred and crossbred hens (Star et al., 2007a; Sun et al., 2011). NAb are defined as antibodies present in individuals in the absence of immunization, vaccination or previous infection (Baumgarth et al., 2005). NAb are an important component of the first line of defense and can prevent (Tomer et al., 1988) and combat infection (Ochsenbein et al., 1999; Baumgarth et al., 2005). Previous research showed that KLH binding NAb titers (Sun et al., 2013a; Sun et al., 2013b), and rabbit red blood cell binding NAb (Wijga et al., 2009) are heritable in chickens. Considering their role in survival and their heritability, NAb might be a promising trait for selection and breeding for improved general disease resistance. However, the consequences of selection for NAb are on production traits of laying hens are unknown. The aim of the present study was to investigate the phenotypic and genetic correlations between total levels of NAb, the isotypes IgM, and IgG, and production traits in a purebred white leghorn chicken line.

Materials and Methods

Ethics statement

Samples and data were collected according to ISA protocols under the supervision of ISA employees. Samples and data were collected as a routine in a commercial breeding program for layer chickens in The Netherlands. Samples and data were routinely collected on a

breeding nucleus of ISA for breeding purposes only and not for commercial production. ISA complies with Dutch law on welfare and health of animals (Gezondheids- en welzijnswet voor dieren), which governs procedures and practices in such breeding programs.

Study Population

The purebred white leghorn chicken line (in other work referred to as "WA") is a layer chicken line selected mainly for egg production. In addition, egg characteristics are included in the breeding goal.
Plasma of the studied chicken population was collected at 15 weeks of age (males) or 19 weeks of age (females) and stored at -20°C until use. In total 2,385 hens with NAb titer measurements and production observations were included in this study.
The studied chicken population originated from 314 dams. Chickens hatched at three subsequent moments with a 2 week interval (dam age: 50-60 weeks), and were group housed with 12-14 males or 15-20 females until wk 18 of age, and subsequently individually housed. Chickens received a standard rearing diet 1 until wk 8, a standard rearing diet 2 from wk 8 until wk 16, and a standard laying diet from wk 16 until end of laying period. Feed was provided ad libitum and water was provided ad libitum (wk 0-16) or 15.5 h per day (wk 16 until end). The light regime was 24 h light per day at hatch, and was weekly, gradually reduced to 9 h light per day at 11 weeks of age. From 19 weeks of age, the light regime was weekly extended with 1 h until 15 h light per day.
The chickens received obligatory vaccinations against Marek's disease (d 1 intramuscular (i.m.)), infectious bronchitis (d 1, d 12-14, wk 10, wk 12 via spray; wk 16 i.m.), Newcastle disease (d 13, d 42, wk 12 via spray; wk 16 i.m.), infectious bursal disease (d 25 via spray; wk 16 i.m.), chicken anemia virus (wk 16 via water), fowl pox (wk 16 by wing web injection), and avian encephalomyelitis (wk 16 by wing web injection).

Production Traits

Body weight was recorded once between 35 and 40 weeks of age. Daily egg production was recorded and summed between 17 and 24 weeks of age, 25 and 34 weeks of age, and 35 and 56 weeks of age. Egg weight was based on the average weight of the eggs laid on 7 subsequent days between 25 to 28 weeks of age, 35 to 56 weeks of age, and 57 to 83 weeks of age. Eggs less than 30 g and eggs over 90 g were excluded, as well as double-yolk eggs. Egg breaking strength was recorded as the average breaking strength of all eggs (maximum 6 eggs) laid during a 6 day-period between 35 to 56 weeks of age, and 57 to 83 weeks of age. Egg breaking strength was determined using the Eggshell tester (FUTURA, Lohne, Germany) according to manufacturer's manual. Early eggshell color was based on the average of the three first laid eggs. Early eggshell color was measured with a Konica Minolta spectrophotometer, which was recorded as a color value on a color measurement scale (LAB color space). Feed conversion ratio (FCR) was recorded between 33 and 43 weeks of age and

calculated as the feed intake (g) divided by the egg mass produced (g) during a measuring period of three consecutive weeks.

Natural antibodies binding KLH

Titers of total immunoglobulins binding KLH (IgT) and the immunoglobulin isotypes IgM and IgG binding KLH were determined in individual plasma samples by an indirect two-step ELISA as described by Sun et al. (2011). Briefly, flat-bottomed, 96-well medium binding plates (Greiner Bio-One, Alphen a/d Rijn, The Netherlands) were coated with 2 µg/mL KLH (Sigma-Aldrich, St. Louis, MO, USA) in 100 µL coating buffer (5.3 g/L Na_2CO_3, and 4.2 g/L $NaHCO_3$, pH 9.6), and incubated at 4°C overnight (o/n). After washing with tap water containing 0.05% TWEEN 20 for 6 s, plates were tapped dry. Plasma samples were 1:10 pre-diluted and stored at 4°C until use the next day or frozen until use. Plasma samples were 4 stepwise diluted with dilution buffer (phosphate buffered saline (PBS; 10.26 g/L $Na_2HPO_4 \cdot H_2O$, 2.36 g/L KH_2PO_4, and 4.50 g/L NaCl, pH 7.2) containing 0.05% TWEEN 20 and 0.5% normal horse serum). Duplicate standard positive plasma samples (a pool of approximately half of the males) were stepwise diluted with dilution buffer. The plates were incubated for 1.5 h at 23°C. After washing, plates were incubated with 1:20,000-diluted rabbit-anti-chicken IgG heavy and light chain (IgT) labeled with horse radish peroxidase (Cat# A30-107P, RRID:AB_67386), or 1:20,000-diluted goat-anti-chicken IgM labeled with horse radish peroxidase (Cat# A30-102P, RRID:AB_66857), or 1:40,000-diluted goat-anti-chicken IgG(Fc) labeled with horse radish peroxidase (Cat# A30-104P, RRID:AB_66843) (all polyclonal antibodies from Bethyl Laboratories, Montgomery, TX, USA; see also www.antibodyregistry.org), and incubated for 1.5 h at 23°C. After washing, binding of the antibodies to KLH was visualized by adding 100 µL substrate buffer (containing reverse osmosis purified water, 10% tetramethylbenzidine buffer (15.0 g/L sodium acetate, and 1.43 g/L ureumperoxide, pH 5.5), and 1% tetramethylbenzidine (8 g/L TMB in DMSO)). After 10 min, the reaction was stopped with 50 µL of 1.25 M H_2SO_4. Extinctions were measured with a Multiskan Go (Thermo scientific, Breda, The Netherlands) at 450 nm. Antibody titers were calculated based on log2 values of the dilutions that gave extinction closest to 50% of E_{MAX}, where E_{MAX} represents the mean of the highest extinction of the standard positive plasma samples, thereby partly correcting for plate differences.

Statistical Analyses

The following linear animal model was used for estimating variance components for IgT, IgM, and IgG titers binding KLH:

$$y_{ij} = \mu + P_i + a_j + e_{ij} \qquad [1]$$

where y_{ij} is the IgT, IgM, or IgG titer, μ is the overall mean, P_i is the fixed effect of plate on which a sample was analyzed (i = 1-188), a_j is the random additive genetic effect of the j^{th} animal assumed to be ~N(0, $\mathbf{A}\sigma_a^2$), and e_{ij} is the residual term assumed to be ~N(0, $\mathbf{I}\sigma_e^2$). Note

that the plate effect corrects also for other (confounding) effects on the samples, such as sex, storage, and analyses effects.

The model for the production traits was

$$y_{ij} = \mu + H_i + a_j + e_{ij} \qquad [2]$$

where y_{ij} is a production trait, μ is the overall mean, H_i is the fixed effect of moment of hatch (i = 1-3), a_j is the random additive genetic effect of the j^{th} animal, and e_{ij} is the residual term. Assumed (co)variance structures of the random model terms are $\mathbf{A}\sigma_a^2$ and $\mathbf{I}\sigma_e^2$, in which **A** is the additive genetic relationship matrix, σ_a^2 is the additive genetic variance, **I** is an identity matrix, and σ_e^2 is the residual variance. The pedigree used to construct **A** consisted of 4,586 individuals and was based on 7 generations of ancestors.

Heritabilities were calculated as

$$h^2 = \frac{\sigma_a^2}{\sigma_p^2}$$

and phenotypic variance was $\sigma_p^2 = \sigma_a^2 + \sigma_e^2$, where σ_a^2 is the additive genetic variance, and σ_e^2 is the residual variance. The likelihood ratio test was used to test whether estimated heritabilities were significant different from 0, comparing the univariate model to an univariate model in which additive genetic variance was fixed at a small value of 0.0001. The likelihood ratio test was -2ln(Λ(x)), with

$$\Lambda(x) = \frac{\max\,[\,L_0|x]}{\max\,[\,L_1|x]}$$

where L_0 is the likelihood under the null hypothesis with the additive genetic variance fixed at 0.0001, L_1 is the likelihood under the alternative hypothesis without variance components constrained, and x is the given data set. Significance was assessed assuming that the likelihood ratio follows a χ_1^2 distribution.

To test for an maternal environmental effect on production or immune traits, model [1] and [2] were extended with d_k, a random effect of the k^{th} dam, where effects of d_k are assumed to be ~N(0, $\mathbf{I}\sigma_m^2$) with **I** as an identity matrix, and σ_m^2 as the maternal variance. The likelihood ratio test was used to test for significance of maternal environmental effects, comparing model [1] or model [2] without maternal effects with the same model with maternal effects. When maternal effects were significant, the heritability and the contribution of the maternal effect was calculated as

$$h^2 = \frac{\sigma_a^2}{\sigma_p^2} \quad \text{and} \quad m^2 = \frac{\sigma_m^2}{\sigma_p^2}$$

and phenotypic variance was $\sigma_p^2 = \sigma_a^2 + \sigma_m^2 + \sigma_e^2$, where σ_a^2 is the additive genetic variance, σ_m^2 is the maternal variance, and σ_e^2 is the residual variance.

Phenotypic and genetic correlations between NAb titers and production traits were estimated based on bivariate analyses using models [1] and [2] or the alternative models in case of a significant maternal effect. A Fishers *r* to *z*-transformation was used to test whether phenotypic correlations were different from 0, with test statistic

$$z = \frac{1}{2}\ln\left(\frac{1 + r}{1 - r}\right)$$

where r is the estimated phenotypic correlation and z follows a normal distribution with standard deviation $1/\sqrt{n-3}$, where n is the number of chickens used to estimate the phenotypic correlation. The likelihood ratio test was used to test whether genetic correlations were different from 0, comparing bivariate models in which no (co)variances were fixed with a bivariate model in which the additive genetic covariance was fixed at 0.
All statistical analyses were performed using ASREML 4 (Gilmour et al., 2014).

Results

The number of observations, mean, standard deviation and coefficient of variation of the NAb titers and the production traits are listed in Table 1. All traits are approximately normally distributed, except for cumulative egg production between 25 to 34 wks of age and 35 to 56 wks of age. The data of these two traits were skewed to the right, with a skewness of -10.17 and a kurtosis of 112 for egg production between 25 and 34 wks of age, and a skewness of -8.73 and a kurtosis of 91 for egg production between 35 and 56 wks of age. Average body weight of the hens between 35 and 40 wks of age was 1.60 kg. Average egg weight over the total production period varied from 53.10 g in early life production (17 to 24 wks of age), to

Table 1 (*IgA included*). Number of individuals (n), mean, SD and coefficient of variation (CV) of total natural antibody (IgT) titers, and titers of isotype IgM, IgA and IgG binding keyhole limpet hemocyanin (KLH) and production traits of purebred white laying chickens.

Trait	n	Mean	SD	CV
IgT[1]	3,689	7.2	1.4	0.19
IgM[1]	3,689	7.5	1.3	0.17
IgA[1]	3,547	6.5	1.3	0.20
IgG[1]	3,689	6.3	1.6	0.25
Body weight (kg)				
wk 35 - 40	2,347	1.60	0.13	0.08
Egg production[2]				
wk 17 - 24	2,384	23.44	8.60	0.37
wk 25 - 34	2,368	68.40	5.82	0.09
wk 35 - 56	2,273	148.00	12.80	0.09
Egg weight (g)				
wk 25 - 28	2,369	53.10	2.78	0.05
wk 35 - 56	2,262	58.07	3.13	0.05
wk 57 - 83	2,149	57.33	3.35	0.06
Breaking strength (kg)				
wk 35 - 56	2,254	4.17	0.44	0.11
wk 57 - 83	2,141	3.64	0.49	0.13
Eggshell color[3]	2,316	87.27	2.40	0.03
Feed conversion ratio[4]				
wk 33 - 43	2,341	1.98	0.21	0.11

[1] Natural antibody titers were measured on females (n = 2,392) and males (n = 1,297).
[2] Egg production was measured as the number of eggs laid in the indicated period.
[3] Eggshell color was measured on the first three eggs laid.
[4] Feed conversion ratio was calculated as the amount of feed consumed divided by the egg mass produced.

58.07 g as maximum during peak production (25 to 34 wks of age), and 57.33 g during later life (35 to 56 wks of age). Egg production peaked in time and then reduced, but egg breaking strength decreased in time.

Heritabilities. Heritabilities and maternal effects for NAb and production traits are shown in Table 2. For most production traits, no significant maternal effects were present except for egg production later in life. A high heritability of 0.64 was estimated for bodyweight, and 0.55 for early eggshell color. FCR heritability was estimated at 0.32. Heritabilities for egg weight tended to be slightly lower at older age: 0.65 for egg weight between 25 and 28 wks of age, 0.63 for egg weight between 35 and 56 wks of age, and 0.54 for egg weight between 57 and 83 wks of age. Also heritabilities estimates for breaking strength tended to decrease in time: 0.44 for breaking strength between 35 and 65 wks of age, and 0.37 for breaking strength between 57 and 83 wks of age. Egg production heritability between 17 and 24 wks

Table 2 *(IgA included)*. Estimates of heritabilities, phenotypic variance and the fraction explained by significant maternal environmental effects of total natural antibody (IgT) titers, and titers of isotype IgM, IgA, and IgG binding keyhole limpet hemocyanin (KLH) and production traits of purebred white laying chickens. Associated standard errors are shown in parentheses.

Trait[1]	σ_p^2	h^2	m^2
IgT[2]	1.86 (0.05)	0.12 (0.03)	NS
IgM[2]	1.27 (0.04)	0.14 (0.05)	0.06 (0.02)
IgA[2]	1.50 (0.04)	0.10 (0.02)	NS
IgG[2]	1.68 (0.04)	0.07 (0.02)	NS
Body weight (kg)			
wk 35 - 40	0.02 (0.01)	0.64 (0.06)	NS
Egg production[3]			
wk 17 - 24	47 (2)	0.37 (0.05)	NS
wk 25 - 34	36 (1)	0.00[4] (0.00)	0.04 (0.01)
wk 35 - 56	164 (5)	0.00[4] (0.00)	0.03 (0.01)
Egg weight (g)			
wk 25 - 28	8 (0.38)	0.65 (0.06)	NS
wk 35 - 56	10 (0.48)	0.63 (0.06)	NS
wk 57 - 83	12 (0.51)	0.54 (0.06)	NS
Breaking strength (kg)			
wk 35 - 56	0.19 (0.01)	0.44 (0.06)	NS
wk 57 - 83	0.24 (0.01)	0.37 (0.05)	NS
Eggshell color[5]	5.80 (0.25)	0.55 (0.06)	NS
Feed conversion ratio[6]			
wk 33 - 43	0.04 (0.002)	0.32 (0.05)	NS

[1] σ_p^2 is the phenotypic variance ($\sigma_a^2 + \sigma_e^2$ or $\sigma_a^2 + \sigma_m^2 + \sigma_e^2$, when maternal environmental effects were significant), h^2 is the heritability ($h^2 = \sigma_a^2 / \sigma_p^2$), and m^2 is the fraction explained by significant maternal environmental effects ($m^2 = \sigma_m^2 / \sigma_p^2$).

[2] Results taken from Berghof et al. (submitted).

[3] Egg production was measured as the number of eggs laid in the indicated period.

[4] Heritability was not significantly different from 0.

[5] Eggshell color was measured on the first three eggs laid.

[6] Feed conversion ratio was calculated as the amount of feed consumed divided by the egg mass produced.

of age was moderate: 0.37. Egg production heritabilities later in life were not significantly different from 0, but significant maternal environmental effects were present.

Phenotypic and Genetic Correlations. An overview of genetic and phenotypic correlations between the different NAb isotypes and the production traits is given in Tables 3 and 4. In general, few genetic correlations were significant and almost half of the phenotypic correlations was significant. However, genetic correlations were stronger than phenotypic correlations.

Genetic and phenotypic correlations between body weight and NAb titers were very low, but a significant phenotypic correlation with IgM titers (-0.05) was estimated. Positive phenotypical correlations between egg production (17 to 24 wks of age) and NAb titers were estimated. However, no significant genetic correlations were found. IgT (0.04) and IgM (0.15) were significant phenotypically correlated to egg production. Egg weight showed consistently negative correlations with titers of IgT, IgM, and IgG. Egg weight and IgM titers were significant phenotypically correlated throughout the whole production period: -0.06 for 25 to 28 wks of age, -0.07 for 35 to 56 wks of age, and -0.04 for 57 to 83 wks of age. In addition, egg weight between 25 and 28 wks of age was significant phenotypically correlated to IgT (-0.04), and tended to be phenotypically correlated to IgG (-0.03). No significant genetic correlations between egg weight and NAb titers were detected. Egg breaking strength had low genetic correlations with IgM (-0.02 and -0.07), but moderate genetic correlations with IgT (-0.19 and -0.14), and IgG (-0.29 and -0.19). However, only breaking strength

Table 3 *(IgA included)*. Estimates of genetic correlations between total natural antibody (i.e., IgT) titers, titers of isotype IgM, IgA and IgG binding KLH at 19 wks of age, and production traits of purebred white laying chickens. Standard errors are shown in parentheses.

Trait	IgT	IgM	IgA	IgG
Body weight (kg)				
wk 35 - 40	0.04 (0.13)	-0.003 (0.14)	-0.04 (0.13)	0.03 (0.15)
Egg production[1]				
wk 17 - 24	0.06 (0.14)	0.18 (0.15)	0.02 (0.14)	0.10 (0.16)
Egg weight (g)				
wk 25 - 28	-0.10 (0.13)	-0.23 (0.14)	-0.05 (0.13)	-0.18 (0.15)
wk 35 - 56	-0.05 (0.13)	-0.18 (0.14)	-0.06 (0.13)	-0.16 (0.15)
wk 57 - 83	-0.05 (0.13)	-0.11 (0.15)	-0.06 (0.14)	-0.11 (0.15)
Breaking strength (kg)				
wk 35 - 56	-0.19 (0.14)	-0.02 (0.15)	0.14 (0.14)	-0.29† (0.16)
wk 57 - 83	-0.14 (0.14)	-0.07 (0.16)	0.02 (0.14)	-0.19 (0.16)
Eggshell color[2]	0.03 (0.13)	-0.05 (0.15)	-0.14 (0.13)	0.08 (0.15)
Feed conversion ratio[3]				
wk 33 - 43	0.27† (0.13)	0.21 (0.15)	0.26† (0.14)	0.33* (0.15)

[1] Results for wk 25 - 34 and wk 35 - 56 are not given because of convergence errors. Egg production was measured as the number of eggs laid in the indicated period.

[2] Eggshell color was measured on the first three eggs laid.

[3] Feed conversion ratio was calculated as the amount of feed consumed divided by the egg mass produced.

* Different from 0 ($P < 0.05$)

† Different from 0 ($P < 0.10$)

between 35 to 56 wks of age and IgG tended to be significantly correlated (-0.29). Very low phenotypical correlations (-0.002 to -0.04) between breaking strength and NAb titers were observed. Breaking strength between 57 and 83 wks of age tended to be significant phenotypically correlated to IgM (-0.04), and to IgG (-0.03), but not to IgT. Early eggshell color was not correlated to any KLH binding NAb type. FCR tended to be genetically correlated to IgT (0.27), and was significant phenotypically correlated to IgT (0.04). FCR was also significant genetically correlated to IgG (0.33), and was significant phenotypically correlated to IgG (0.04). IgM had a moderate genetic correlation (0.21) with FCR, but was not significant.

Discussion

To the best of our knowledge this is the first large scale study on genetic relations between production traits and NAb in a purebred white leghorn chicken line.

Production data and heritabilities of production traits were comparable to literature (Szwaczkowski, 2003) and characteristics of the studied chicken line by ISA (T. van de Braak, ISA; personal communication). Cumulative egg production between 25 to 34 wks of age and from 35 to 56 wks of age had very low heritabilities. The studied line is highly productive: almost every hen laid the maximum of one egg per day. Therefore, there is little genetic variation left for this trait in this highly selected line, resulting in heritabilities of 0.

Table 4 *(IgA included)*. Estimates of phenotypic correlations between total natural antibody (i.e., IgT) titers, titers of isotype IgM, IgA and IgG binding KLH at 19 wks of age, and production traits of purebred white laying hens. Standard errors are shown in parentheses.

Trait	IgT	IgM	IgA	IgG
Body weight (kg)				
wk 35 - 40	-0.01 (0.02)	-0.04* (0.03)	0.01 (0.02)	-0.003 (0.02)
Egg production[1]				
wk 17 - 24	0.04* (0.02)	0.15* (0.03)	-0.08* (0.02)	0.003 (0.02)
Egg weight (g)				
wk 25 - 28	-0.04* (0.02)	-0.06* (0.03)	0.03† (0.02)	-0.03† (0.02)
wk 35 - 56	-0.01 (0.02)	-0.07* (0.03)	0.02 (0.02)	-0.02 (0.02)
wk 57 - 83	-0.01 (0.02)	-0.04* (0.03)	0.03† (0.02)	-0.02 (0.02)
Breaking strength (kg)				
wk 35 - 56	-0.002 (0.02)	-0.02 (0.03)	0.05* (0.02)	-0.002 (0.03)
wk 57 - 83	-0.01 (0.02)	-0.03† (0.03)	0.01 (0.02)	0.03† (0.02)
Eggshell color[2]	0.03 (0.02)	0.01 (0.03)	-0.01 (0.02)	0.01 (0.02)
Feed conversion ratio[3]				
wk 33 - 43	0.04* (0.02)	-0.004 (0.03)	0.01 (0.02)	0.04* (0.02)

[1] Results for wk 25 - 34 and wk 35 - 56 are not given because of convergence errors. Egg production was measured as the number of eggs laid in the indicated period.

[2] Eggshell color was measured on the first three eggs laid.

[3] Feed conversion ratio was calculated as the amount of feed consumed divided by the egg mass produced.

* Different from 0 ($P < 0.05$)

† Different from 0 ($P < 0.10$)

Phenotypes for these cumulative egg production traits were not normally distributed, which could have affected the heritability estimates. A Box-Cox transformation (Box et al., 1964) is commonly applied for the analysis of egg production data (Besbes et al., 1993). However, transformation did not solve the problem of non-normality (data not shown). Therefore these traits were excluded from further analyses.

Rauw et al. (1998) reviewed the negative effects of selection for increased production efficiency on health traits and immune competence in broilers, turkeys, pigs, and dairy cattle. However, the effect of selection for production traits on health traits in layers is still unclear. Our findings show that correlated response of previous selection on egg weight, breaking strength, and FCR might have been accompanied by lower NAb titers and thus a decreased natural immune competence. Consumer demands for egg size (egg weight) puts emphasis on selection for a particular optimal egg weight range. Therefore selection on egg weight will likely not have considerably decreased NAb titers. In addition, Star et al. (2007a) found a positive relationship between levels of NAb and the probability to survive a laying period. Also Sun et al. (2011) and Sun et al. (2013b) showed that NAb titers around 20 wks of age were significantly associated with survival in both purebred and crossbred hens. Therefore, selection for survival might have limited a decrease in NAb titers in commercial layer lines. In the current layer line hens were culled based on their breeding values for egg production at several moments during production and therefore NAb titers could not be related to survival.

Selection and breeding in chickens for specific health traits might influence resource allocation (Rauw, 2009). Resource allocation is the distribution of resources amongst the several energy requirements of the living organism, such as growth, reproduction, maintenance, and immunity. Because resources are limited, trade-offs between the different functions are thought to be inevitable (Van der Most et al., 2011; Rauw, 2012). For example, two independent chicken lines (i.e. a white and a brown layer line) selected for high primary antibody responses to sheep red blood cells (SRBC) had lower body weight, decreased egg production, and decreased egg(shell) weight and eggshell thickness than chickens selected for low primary antibody responses to SRBC (Siegel et al., 1980; Siegel et al., 1982; Pinard-Van der Laan et al., 1998; Adriaansen-Tennekes et al., 2009; Albrecht et al., 2012a; Parmentier et al., 2012; Zhao et al., 2012).

The estimated negative relation between NAb titers and egg breaking strength, and between NAb titers and egg weight is in line with the resource allocation theory. This relation between egg weight and immunity was previously described by Adriaansen-Tennekes et al. (2009) and Parmentier et al. (2012) in brown layer chickens. Both studies showed that egg weight differed in lines divergently selected for a primary antibody response to SRBC. Chickens from the low antibody response line produced heavier eggs than chickens of the high antibody response line. This indicates that selection allocated the resources used for egg mass production to NAb production or vice versa. However, in a different experiment on SRBC response in white layer chickens, egg weight was not found to differ between high and low antibody response lines (Siegel et al., 1982; Albrecht et al., 2012a).

Based on resource allocation theory a relation between NAb and efficiency of egg production (FCR) might be expected. FCR was genetically correlated to NAb titers, of which IgG was most pronounced. This suggests that immune reactive birds require more feed for maintenance and production. However, production of antibody molecules only requires a small amount of amino acids and energy as compared to production of eggs (Iseri et al., 2013), suggesting that direct resource allocation between NAb and egg production is unlikely. This is supported by our finding that the phenotypic correlation between NAb titers and FCR is very low. The observed relationship might be explained by the fact that when calculating the FCR, the digestibility of the feed is not taken into account. When the digestibility of dietary nutrients is reduced, the FCR may increase, as more feed is needed to absorb the same amount of amino acids and energy from a particular diet. Digestibility can be influenced by several factors, such as digestive enzyme production, secretion and activity, the absorptive capacity of the epithelium, and the microbial composition of the gut. The latter has often been associated with both intestinal and peripheral immunity (Round et al., 2009; Cerf-Bensussan et al., 2010; Calenge et al., 2013) and is strongly related to the development of the immune system in chickens (Gabriel et al., 2006). In addition, it has been shown that a highly diverse microbial composition in the gut reduces FCR (Stanley et al., 2012). Therefore the found results suggest that an indirect relationship between feed efficiency and the immune system is present.

Even though the resource allocation theory predicts a negative relation between egg production and immunity, we found very small correlations between NAb titers and body weight, and positive phenotypic correlations between levels of IgM and IgG binding KLH and egg production between 17 to 24 wks of age. Sexual maturation of the hens may at least partly explain the relation between NAb levels and early egg production. Egg production between 17 to 24 wks is considered to be an indicator of (early) maturation; hens that start laying early in the period between 17 to 24 wks of age, likely produce a high number of eggs in this period. In layer chickens, little is known about the physiological relationship between sexual maturation and the development of the immune system. During sexual maturation local immunity in the oviduct develops and the frequency of IgM+ cells, and IgG+ cells in the epithelial lining increases (Zheng et al., 1997). This process is likely controlled by estrogen (Zheng et al., 1997). Circulating sex steroids may influence NAb titers, and early egg production. Contradictory, previous studies described that hens selected for a high primary antibody response to SRBC after 29 generations laid their first egg 23 d later than hens selected for low primary specific antibody response to SRBC (Parmentier et al., 2012). This was also observed in a different selection experiment for SRBC antibody response: the high SRBC antibody response line laid their eggs later than the low response line (Albrecht et al., 2012b; Zhao et al., 2012). In addition, the low antibody response line showed to have a longer duration of fertility than the high antibody response line (Albrecht et al., 2012b). Martin et al. (1990) found a realized genetic correlation between age at sexual maturity and antibody response to SRBC of -0.28 in a line selected for low antibody response. However in the line selected for high specific antibody response, no genetic correlation was present

(Martin et al., 1990). This suggests that sexual maturation and immune development are related, but also that SpAb and NAb titers are probably genetically different traits.
Maternal effects did not significantly affect production traits. However previously we found that maternal production traits did affect the estimated maternal environmental effects of IgM titers binding KLH at early age (Berghof et al., submitted). The maternal environmental effect may reflect transgenerational information that prepares and protects the offspring for diseases present in the environment (Berghof et al., 2013). Early eggshell color (first three eggs laid) and breaking strength were identified as factors contributing to maternal effects influencing IgM titers in offspring (Berghof et al., submitted), but these traits were not significantly correlated to IgM in the offspring generation. This suggests that several dam characteristics (e.g. breaking strength, early eggshell color, other) affect IgM anti-KLH NAb titers in the offspring, but there is no direct genetic correlation between the traits breaking strength or early eggshell color and IgM NAb titers.
The present results indicate that the possible consequences for production efficiency need to be taken into account when selective breeding for improved general disease resistance in laying chicken is applied. However, the correlated effect on production traits of selection on NAb is difficult to predict. SNP studies are underway to identify genomic regions underlying titers of NAb binding KLH and several production traits.

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References

See List of references at the end of this thesis.

CHAPTER 4

Genomic region containing Toll-like receptor genes has a major effect on IgM (natural) antibodies in chickens

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Abstract

Natural antibodies (NAb) are antigen binding antibodies present in individuals without a previous exposure to this antigen. Keyhole limpet hemocyanin (KLH)-binding NAb levels were previously associated with survival in chickens. This suggests that selective breeding for NAb may increase survival by means of improved general disease resistance. Genome-wide association studies (GWAS) were performed to identify genes underlying genetic variation in NAb levels. The studied population consisted of 1,628 layer chickens with observations for titers of total KLH-binding NAb (IgT), and the isotypes IgM, IgA, and IgG, and antibody concentrations of total IgM, total IgA, and total IgG, and total antibodies concentration in plasma around 16 weeks of age. GWAS were performed using 57,636 single nucleotide polymorphisms (SNP). One chromosomal region on chromosome 4 was associated with IgT NAb, and total IgM concentration, and especially with IgM NAb. The region of interest was fine mapped by imputing the region of the study population to whole genome sequence, and subsequently performing an association study using the imputed sequence variants. 16 candidate genes were identified, of which *FAM114A1*, Toll-like receptor 1 family member B (*TLR1B*), *TLR1A*, Krüppel-like factor 3 (*KLF3*) showed the strongest associations. SNP located in coding regions of the candidate genes were checked for predicted changes in protein functioning. One SNP (at 69,965,939 base pair) received the maximum impact score from two independent prediction tools, which makes this SNP the most likely causal variant. This SNP is located in *TLR1A*, which suggests a fundamental role of TLR1A on regulation of IgM levels, or B cells biology, or both. This study contributes to increased understanding of (genetic) regulation of NAb levels and total antibody concentrations, and can add to selection for improved general disease resistance in poultry.

Keywords: Natural antibodies, Antibody concentration, Genetic parameter, Heritability, Genome-wide association study, B cell development, B cell maturation, B cell survival

Introduction

Modern poultry production is facing high impact changes in production systems and management (Hodges, 2009; Neeteson-Van Nieuwenhoven et al., 2016). These changes may enhance the risks of infectious disease, because of higher pathogenic pressure. According to FAO statistics, economic losses in poultry production due to diseases was as high as 10-20% of the gross production value in 2014 (FAO, 2014). Only few years ago, diseases were controlled by (preventive, and abundant) use of antibiotics, but this resulted in an increased risk for antibiotic resistance. In addition to development of new vaccines and vaccination strategies, and new feed formulations, other sustainable strategies for obtaining robust populations need to be developed. Selective breeding for an improved immune system might be an important additional strategy to improve general disease resistance (Cheng et al., 2013). Selective breeding for an improved immune system requires a trait that is heritable, easy to measure, and preferentially related to general disease resistance (Berghof et al., 2015). Natural antibody (NAb) titers might be a good candidate trait. NAb are defined as antigen binding antibodies present in individuals without a previous exposure to this antigen. NAb play an essential role in the innate and adaptive immunity against self-antigens and various types of pathogens, and are contributing to healthy aging and disease resistance (Zhou et al., 2007; Panda et al., 2015; Rothstein, 2016). Different NAb isotypes are found: predominantly IgM, but also IgA, and IgG (Baumgarth et al., 2015; Rothstein, 2016). In earlier studies, high NAb levels (especially IgM) binding the overt antigen keyhole limpet hemocyanin (KLH) were related to lower mortality of commercial layer chickens (Star et al., 2007a; Sun et al., 2011; Wondmeneh et al., 2015). NAb levels binding KLH around 16 weeks of age were previously estimated to have heritabilities between 0.07 and 0.14 in white layer chickens (Berghof et al., 2015). In addition, some genomic regions underlying genetic variation of NAb levels were identified by using dedicated single nucleotide polymorphism (SNP) sets with a limit number of SNP (Biscarini et al., 2010; Sun et al., 2013a). However, to the best of our knowledge, no genome-wide association studies (GWAS) for NAb levels in chicken have been reported.

This study describes the first GWAS on NAb in chicken. The studied population consisted of 1,628 purebred White Leghorn chickens with observations for total KLH binding immunoglobulin (IgT), and the isotypes IgM, IgA, and IgG around 16 weeks of age. Identification of associated genomic regions was performed by using 57,636 SNP in a single SNP GWAS. Regions were fine mapped by performing an association study using imputed genotypes based on sequence data with the aim to identify candidate gene(s), and possibly the causal variant(s). A strong association was found for IgM NAb on chromosome 4 near *TLR1A* and *TLR1B*, which suggests a fundamental involvement of TLR on total IgM antibody concentration. Therefore also total antibody (tIgT) concentration, total IgM (tIgM) concentration, total IgA (tIgA) concentration, and total IgG (tIgG) concentration in plasma were measured and used to study the role of the found association on (natural) antibodies. The role of the found association in general disease resistance in chickens is discussed.

Material and Methods

Ethics approval statement

Collection of samples and data was done according to Hendrix Genetics (HG) protocols, under the supervision of HG employees. Samples and data were collected as part of routine data collection in a commercial breeding program for layer chickens in The Netherlands. Samples and data were collected on a breeding nucleus of HG for breeding purposes only, and are a non-experimental, agricultural practice, regulated by the Act Animals, and the Royal Decree on Procedures. The Dutch Experiments on Animals Act does not apply to non-experimental, agricultural practices. An ethical review by the Statement Animal Experiment Committee was therefore not required. No extra discomfort was caused for sample collection for the purpose of this study.

Study population

Samples and data were obtained from a purebred White Leghorn chicken line (in other work referred to as "WA"), which is a layer chicken line selected mainly for egg production, but also for other production traits, e.g. traits related to egg quality. The studied chicken population comprised 1,628 chickens, of which 696 males and 932 females. The study population originated from 112 sires and 288 dams. 437 chickens in the current study were also included in the study of Berghof et al. (2015). Of these 437 chickens, 222 female chickens were included in the study of Van der Klein et al. (2015). The chickens were kept according to standard management of breeding nucleus farms of HG. Further details can be found in Van der Klein et al. (2015).

The chickens received obligatory vaccinations against Marek's disease (1 day of age intramuscular (i.m.)), infectious bronchitis (1 day of age, 12-14 days of age, 10 weeks of age, 12 weeks of age via spray; 16 weeks of age i.m.), Newcastle disease (13 day of age, 42 days of age, 12 weeks of age via spray; 16 weeks of age i.m.), infectious bursal disease (25 days of age via spray; 16 weeks of age i.m.), chicken anemia virus (16 weeks of age via water), fowl pox (16 weeks of age by wing web injection), and avian encephalomyelitis (16 weeks of age by wing web injection).

Blood of the study population was collected once between 15 weeks of age and 22 weeks of age, without anesthesia/analgesia. Samples were collected in four rounds with approximately 1.75 year between the first and last round. No chickens were killed for sample collection. The blood samples were centrifuged, and plasmas and blood cells were collected separately, and stored at −20°C until use.

KLH-binding natural antibody titers

Optical density (OD) of total KLH-binding immunoglobulins (IgT), and the isotypes IgM, IgA, and IgG were determined in individual plasma samples by an indirect two-step ELISA as described by Van der Klein et al. (2015), and Berghof et al. (2015). Briefly, plasma

samples were 1:10 pre-diluted (for IgT, IgM, and IgG analyses), or were 1:5 pre-diluted (for IgA analysis) with dilution buffer (PBS [10.26 g/L $Na_2HPO_4 \cdot H_2O$, 2.36 g/L KH_2PO_4, and 4.50 g/L NaCl; pH 7.2] containing 0.5% normal horse serum, and 0.05% Tween® 20). Pre-dilutions were stored at 4°C until use the next day, or were stored at −20°C until use (with a maximum storage time of three months). Flat-bottomed, 96-well medium binding plates (Greiner Bio-One, Alphen a/d Rijn, The Netherlands) were coated with 2 µg/mL KLH in 100 µL coating buffer (5.3 g/L Na_2CO_3, and 4.2 g/L $NaHCO_3$; pH 9.6) per well, and incubated at 4°C overnight. After washing for 6 s with tap water containing Tween® 20, plates were tapped dry. The 1:10 pre-dilution of the samples were further diluted in the KLH-coated plates with dilution buffer to 1:40, 1:160, 1:640, and 1:2,560 test dilutions for IgT, IgM, and IgG, or 1:10, 1:20, 1:40, and 1:80 for IgA. Duplicate standard positive plasma samples (a pool of male plasmas) were stepwise 1:1 diluted with dilution buffer, and pipetted into the KLH-coated plates. A minimum of 5 samples per plate was maintained to ensure proper adjustment of the titers for plate effects in the statistical analyses. The plates were incubated for 1.5 h at room temperature (20-25°C). After washing, plates were incubated with 1:20,000-diluted rabbit-anti-chicken IgG heavy and light chain (IgT) labeled with horse radish peroxidase (PO) (Cat# A30-107P, RRID:AB_67386), or 1:20,000-diluted goat-anti-chicken IgM labeled with PO (Cat# A30-102P, RRID:AB_66857), or 1:7,500-diluted goat-anti-chicken IgA labeled with PO (Cat# A30-103P, RRID:AB_66833), or 1:40,000-diluted goat-anti-chicken IgG(Fc) labeled with PO (Cat# A30-104P, RRID:AB_66843) (all polyclonal antibodies from Bethyl Laboratories, Montgomery, TX, USA; see also www.antibodyregistry.org), and incubated for 1.5 h at room temperature. After washing, binding of the antibodies to KLH was visualized by adding 100 µL substrate buffer (containing reverse osmosis purified water, 10% tetramethylbenzidine buffer [15.0 g/L sodium acetate, and 1.43 g/L urea hydrogen peroxide; pH 5.5], and 1% tetramethylbenzidine [8 g/L TMB in DMSO]) at room temperature. After approximately 15 min, the reaction was stopped with 50 µL of 1.25 M H_2SO_4. OD were measured with a Multiskan Go (Thermo scientific, Breda, The Netherlands) at 450 nm.

Antibody titers were calculated as described by Frankena (1987) (taken from De Koning et al. (2015)). Briefly, the OD of the duplicate standard positive plasma samples were averaged for each plate. Logit values of the OD per plate were calculated using:

$$logit\ OD\ = \ ln\left(\frac{OD}{(OD_{max} - \ OD)}\right)$$

where OD is the OD of a well, and OD_{max} is the maximum averaged OD of the duplicate standard positive plasma samples. The last positive well (lpw) of the averaged duplicate standard positive plasma sample was set to the sixth dilution. A linear regression line of the logit OD against the respective log_2-dilution values of the averaged duplicate standard positive plasma samples was determined, which resulted in a regression coefficient β. Titers of the plasma samples per plate were calculated using:

$$titer\ = \frac{logit\ OD_{lpw} - \ \left(logit\ OD_{sample} - \ \beta \times log_2\left(dilution_{sample}\right)\right)}{\beta}$$

were logit OD_{lpw} is the estimated logit OD at the lpw calculated with the estimated linear regression function using the $\log_2$-dilution value of that well, logit OD_{sample} is the logit OD calculated of the OD closest to 50% of OD_{max} for a plasma sample of an individual (OD_{sample}), β is the regression coefficient of the estimated linear regression function of the averaged duplicate standard positive plasma samples, and $\log_2(\text{dilution}_{sample})$ is the $\log_2$-dilution value at which OD_{sample} occurred, as described by De Koning et al. (2015).
The total number of observations was 1,625 for IgT NAb, 1,627 for IgM NAb, 1,608 for IgA NAb, and 1,623 for IgG NAb (see Table 1).

Total antibody concentrations

Total concentrations (µg/ml) of the immunoglobulin isotypes IgM (tIgM), IgA (tIgA), and IgG (tIgG) were determined in individual plasma samples by an indirect sandwich ELISA according to manufacturer's protocols (IgM: Cat# E30-102; IgA: Cat# E30-103; IgG: Cat# E30-104; all from Bethyl Laboratories) with minor additions: 1) Plasma samples were 1:5 pre-diluted, and step-wise diluted to 1:500 (for tIgA), 1:5,000 (for tIgM), and 1:50,000 (for tIgG) with Sample/Conjugate Diluent. 2) Flat-bottomed, 96-well medium binding plates (Greiner Bio-One) were used for the ELISA. 3) Washing procedure consisted of washing each well 2 times with 200 µl, and subsequently 3 times with 100 µl Wash Solution. Plates were emptied and tapped dry in between and at the end. 4) 100 µl ELISA Blocking Solution was added to each well. 5) A minimum of 5 samples per plate was maintained to ensure proper adjustment of antibody concentrations for plate effects in the statistical analyses. 6) Two duplicate standard positive plasma samples (obtained from Bethyl Laboratories) were used per plate. 7) Enzyme Substrate (TMB) and ELISA Stop Solution were made and applied as specified for 'KLH-binding natural antibody titers' (see above). 8) OD were measured with a Multiskan Go. 9) All samples on a plate were corrected for the average background OD of the two blanks. 10) SoftMax® Pro 7.0 build 226962 (Free Trial) was used for generating a 4-parameter logistic curve fit based on the averages of the two standard ranges, and calculating isotype concentrations per sample.
The total concentration (µg/ml) of antibodies (tIgT) was calculated by summing the concentrations for tIgM, tIgA, and tIgG. tIgT, tIgM, tIgA, and tIgG were $\log_{10}$-tranformed to normalize the data for statistical analyses. The total number of observations was 1,573 for tIgT, 1,619 for tIgM, 1,586 for tIgA, and 1,615 for tIgG (see Table 1).

Genotypes

DNA was extracted from the collected blood cells. The study population was genotyped for a 2,740 single nucleotide polymorphism (SNP) set (n = 488) (Illumina, San Diego, CA, USA), or for a 52,232 SNP set from which 11,173 SNP were used (n = 1,140) (Illumina). Both sets were imputed with Beagle 4.0 (Browning et al., 2007) (accuracy ≥ 97%) to a 57,636 SNP set, based on approximately 120 key ancestors of this chicken line. The SNP distribution

over the chicken chromosomes (based on Gallus_gallus-5.0) can be found in Supplementary Table 1.

Quality control was applied in four steps: 1) All monomorphic SNP were removed. 2) SNP with at least 1 genotype class with less than 9 chickens with phenotypic observations (~0.5% of the population) were removed. 3) SNP on autosomes with a strong deviation from Hardy Weinberg Equilibrium (χ_1^2-value $\geq$ 600) were removed. 4) SNP on the allosome Z with a strong deviation from Hardy Weinberg Equilibrium (χ_1^2-value $\geq$ 600) in males were removed for the whole population. These criteria were chosen to exclude SNP which are likely to have a high rate of genotyping error and to exclude very low frequency SNP from the data set (Hayes et al., 2009).

Genetic parameters

The additive genetic variances (σ_a^2) and the maternal environmental variance (σ_m^2) for KLH-binding NAb titers were previously estimated in a similar, but larger population of this chicken line (Berghof et al., 2015) (see Table 1). The additive genetic variances (σ_a^2), and heritabilities for tIgT, tIgM, tIgA, and tIgG concentrations were estimated with model [1], in a similar approach as for the NAb titers described by Berghof et al. (2015).

Model [1] was:

$$y_{ij} = \mu + P_i + \beta_1 * Age_{ij} + id_j + e_{ij} \qquad [1],$$

where y_{ij} is the tIgT concentration, tIgM concentration, tIgA concentration, or tIgG concentration, μ is the overall mean, P_i is the fixed effect of plate on which a sample was analyzed (i = 1-26 for tIgM, and tIgA, or i = 1-27 for tIgG, and tIgT), Age_{ij} is the covariate describing the effect of age at sampling (in weeks) with regression coefficient β_1, id_j is the random additive genetic effect of the j^{th} chicken assumed to be distributed as ~N(0,$\mathbf{A}\sigma_a^2$), and e_{ij} is the residual term assumed to be distributed as ~N(0,$\mathbf{I}\sigma_e^2$). Assumed (co)variance structures of the random model terms are $\mathbf{A}\sigma_a^2$, and $\mathbf{I}\sigma_e^2$, in which **A** is the additive genetic relationship matrix, σ_a^2 is the additive genetic variance, **I** is an identity matrix, and σ_e^2 is the residual variance. The pedigree used to construct **A** consisted of 2,537 individuals, and was based on a minimum of 3 generations of ancestors for each individual in the study population. The estimated plate effect also corrects for (confounded) effects on the samples, such as sex, and storage.

Model [1] or model [1] extended with a random maternal environmental effect (for IgM NAb only, see Berghof et al. (2015)), were also used to estimate genetic and phenotypic correlations with bivariate analyses, as described by Berghof et al. (2015).

The statistical analyses were performed using ASReml® 4.1 (Gilmour et al., 2014).

Genome-wide association studies

A single-SNP linear animal model was used for studying KLH-binding IgT, IgM, IgA, and IgG NAb titers, and tIgT, tIgM, tIgA, and tIgG concentrations associations.

All traits, except IgM NAb, were analyzed using model [2a]:

$$y_{ijk} = \mu + P_i + \beta_1 * Age_{ijk} + SNP_j + id_k + e_{ijk} \quad [2a],$$

and IgM NAb was analyzed using model [2b]:

$$y_{ijkl} = \mu + P_i + \beta_1 * Age_{ijkl} + SNP_j + id_k + d_l + e_{ijkl} \quad [2b],$$

where y_{ijk} is the IgT NAb titer, IgA NAb titer, IgG NAb titer, tIgT concentration, tIgM concentration, tIgA concentration, or tIgG concentration, y_{ijkl} is the IgM NAb titer, μ is the overall mean, P_i is the fixed effect of plate on which a sample was analyzed (i = 1-91 for IgT NAb, IgM NAb, and IgG NAb, i = 1-100 for IgA NAb, i = 1-27 for tIgT, and tIgG, or i = 1-26 for tIgM, and tIgA), Age_{ijk} and Age_{ijkl} are the covariates describing the effect of age at sampling (in weeks) with regression coefficient β_1, SNP_j is the fixed effect of genotype class j (j = AA, AB, or BB) of the analyzed SNP, id_k is the random additive genetic effect of the k^{th} chicken assumed to be distributed as $\sim N(0,\mathbf{A}\sigma_a^2)$, d_l is the random effect of the l^{th} dam (IgM NAb only) assumed to be distributed as $\sim N(0,\mathbf{I}\sigma_m^2)$, and e_{ijk} and e_{ijkl} are the residual terms assumed to be distributed as $\sim N(0,\mathbf{I}\sigma_e^2)$. Assumed (co)variance structures of the random model terms are $\mathbf{A}\sigma_a^2$, and $\mathbf{I}\sigma_e^2$ (for all), and $\mathbf{I}\sigma_m^2$ (for IgM NAb only), in which **A** is the additive genetic relationship matrix, σ_a^2 is the additive genetic variance, **I** are identity matrices, σ_e^2 is the residual variance, and σ_m^2 is the maternal environmental variance (for IgM NAb only).

The variances for random factors were fixed to values obtained from analyses from models without a SNP effect: The additive genetic variances (σ_a^2) and the maternal environmental variance (σ_m^2) for NAb titers were fixed to values estimated by Berghof et al. (2015) in a similar population of this chicken line. These estimates are more accurate, because the studied population was bigger. The additive genetic variances (σ_a^2) for tIgT, tIgM, tIgA, and tIgG concentrations were estimated with model [1].

The pedigree used to construct **A** consisted of 2,537 individuals, and was based on a minimum of 3 generations of ancestors for each individual in the study population. The estimated plate effect also corrects for (confounded) effects on the samples, such as sex, and storage.

The statistical analyses were performed using ASReml® 4.1 (Gilmour et al., 2014).

P-values of SNP effects were checked for inflation (Devlin et al., 1999), which was expressed as the inflation factor λ (Lopes et al., 2014). λ was estimated using the "estlambda()" function of the R package "GenABEL" (Aulchenko et al., 2007). Genomic control was applied when $\lambda \geq 1.1$ (WTCCC (2007), taken from Duijvesteijn et al. (2014)) by dividing the test statistics (F-values) by λ, and subsequently recalculating the p-values. A genome-wide false discovery rate (FDR) was estimated based on observed p-values using the R package "qvalue" (Storey et al., 2015). A FDR ≤ 0.05 was used to indicate significant associations, and a FDR ≤ 0.20 was used to indicate suggestive associations. The lead SNP (SNP with highest $-\log_{10}$(p-value)) variance as a percentage of the additive genetic variance within an associated region was estimated as $\frac{\sigma_{SNP}^2}{\sigma_a^2} * 100\%$, where σ_{SNP}^2 was calculated based on estimated SNP effects from model [2a] for all except IgM NAb or model [2b] for IgM NAb

and the genotypic frequencies. Significant differences between genotype classes were tested with a T-test using the "!TDIFF" statement in ASReml® 4.1 (Gilmour et al., 2014). A p-value ≤ 0.05 was considered to be significant, and a p-value ≤ 0.10 was considered to be suggestive.

Fine mapping and identification of possible causal variant

The chickens were imputated to full genome sequence in two steps based on the full genome sequences of 70 key ancestors of this chicken line. Imputation was done with Beagle 4.0 (Browning et al., 2007) (accuracy ≥ 88%) by imputing the 57,636 SNP set (for the 2,740 SNP set) or the 52,232 SNP set to full genome sequence with an average sequence depth of 12.4 (and standard deviation of 2.1).
Associated genomic regions were selected for fine mapping if at least 1 SNP was significant (FDR ≤ 0.05). Fine mapping regions were defined based on the last significant SNP on the border of the significantly associated regions. The fine mapping regions consisted of the first SNP outside the significantly associated regions minus 5 Mbp and plus 5 Mbp.
Quality control of the imputed SNP that were used in the fine mapping studies was applied in four steps as described above. Statistical analyses for the selected regions were performed as described above. Genomic control was applied using λ values from previous analyses, to be able to compare between analyses.
SNP of candidate genes in the fine mapped regions were checked for amino acid substitutions and predicted changes in protein functioning as a result of the nucleotide change in coding regions by using the Ensembl Variant Effect Predictor (McLaren et al., 2016) including the integrated SIFT option (Sim et al., 2012), and by using PolyPhen-2 (Adzhubei et al., 2010).

Results

In total 1,628 chickens, of which 696 males and 932 females, were in the study population. Descriptive statistics and genetic parameters are shown in Table 1. Mean titers for KLH-binding NAb were 5.6 for total levels (IgT), 5.8 for IgM, 5.4 for IgA, and 5.5 for IgG. Mean concentrations for total antibodies were 8,484 μg/ml for tIgT, 355 μg/ml for tIgM, 314 μg/ml for tIgA, and 7,856 μg/ml for tIgG. Heritabilities for total antibody concentrations were estimated to be 0.08 for tIgT, 0.23 for tIgM, 0.22 for tIgA, and 0.06 for tIgG. Maternal environmental effects were not significant for any of the total antibody concentrations. Age was not significant for tIgT, and tIgG, but was kept in the model for all analyses. Heritabilities and maternal environmental variances (σ^2_m) for NAb were previously estimated in a similar, but larger population of this chicken line (see Table 1) (Berghof et al., 2015).
Genetic and phenotypic correlations between the corresponding types of NAb titers and total antibody concentrations are shown in Table 2. Genetic correlations were: −0.27 for IgT NAb/tIgT, 0.91 for IgM NAb/tIgM, 0.38 for IgA NAb/tIgA, and −0.61 for IgG NAb/tIgG. Phenotypic correlations were: 0.03 for IgT NAb/tIgT, 0.41 for IgM NAb/tIgM, 0.26 for

Table 1. Descriptive statistics of KLH-binding natural antibody (NAb) titers and total antibody concentrations (µg/ml) in a White Leghorn chicken population around 16 weeks of age, showing: total KLH-binding NAb (IgT) titer, KLH-binding IgM NAb (IgM) titer, KLH-binding IgA NAb (IgA) titer, KLH-binding IgG NAb (IgG) titer, total antibody (tIgT) concentration, total IgM (tIgM) concentration, total IgA (tIgA) concentration, and total IgG (tIgG) concentration. The table shows: the number of chickens with observation for each trait, the mean and SD of the observations, the range of the observations (5th percentile - 95th percentile), the heritability (h^2) and SE, and the maternal environmental effect (m^2).

	KLH-binding natural antibody titers				Total antibody concentrations (µg/ml)			
	IgT	IgM	IgA	IgG	tIgT[a]	tIgM	tIgA	tIgG
n	1,625	1,627	1,608	1,623	1,573	1,619	1,586	1,615
Mean (SD)	5.6 (1.3)	5.8 (1.1)	5.4 (1.2)	5.5 (1.3)	8,484 (3,741)	355 (201)	314 (143)	7,856 (3,679)
Range[b]	3.6 - 7.7	4.0 - 7.6	3.5 - 7.4	3.5 - 7.8	4,071 - 15,003	142 - 701	157 - 585	3,530 - 14,169
h^2 (SE)	0.12[c]	0.14[c]	0.10[c]	0.07[c]	0.08 (0.04)[d]	0.23 (0.05)[d]	0.22 (0.05)[d]	0.06 (0.03)[d]
m^2	NS[c]	0.06[c]	NS[c]	NS[c]	NS[d]	NS[d]	NS[d]	NS[d]

[a] tIgT = tIgM + tIgA + tIgG

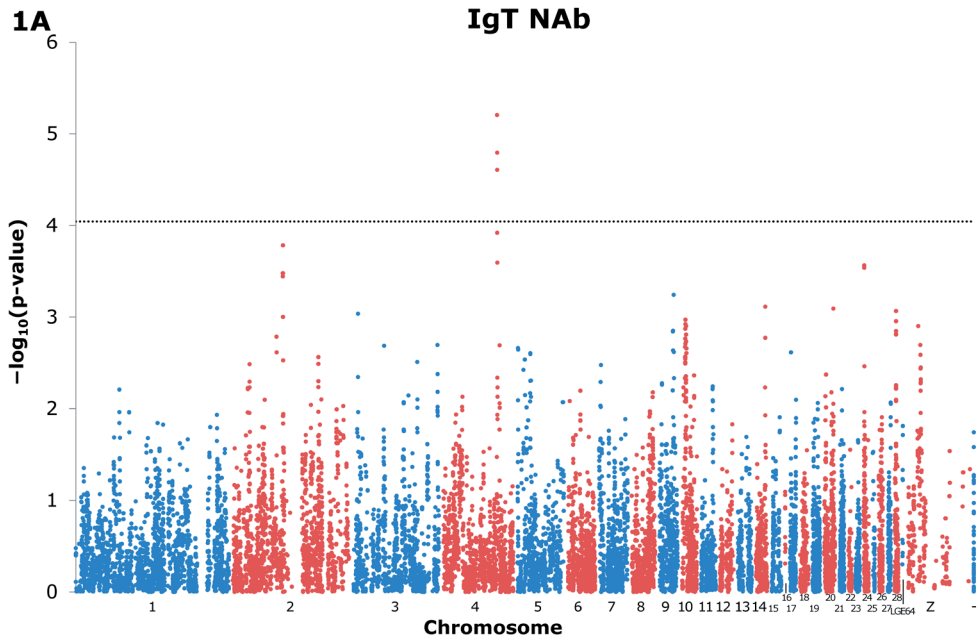
[b] Range shows 5th percentile and 95th percentile, respectively.

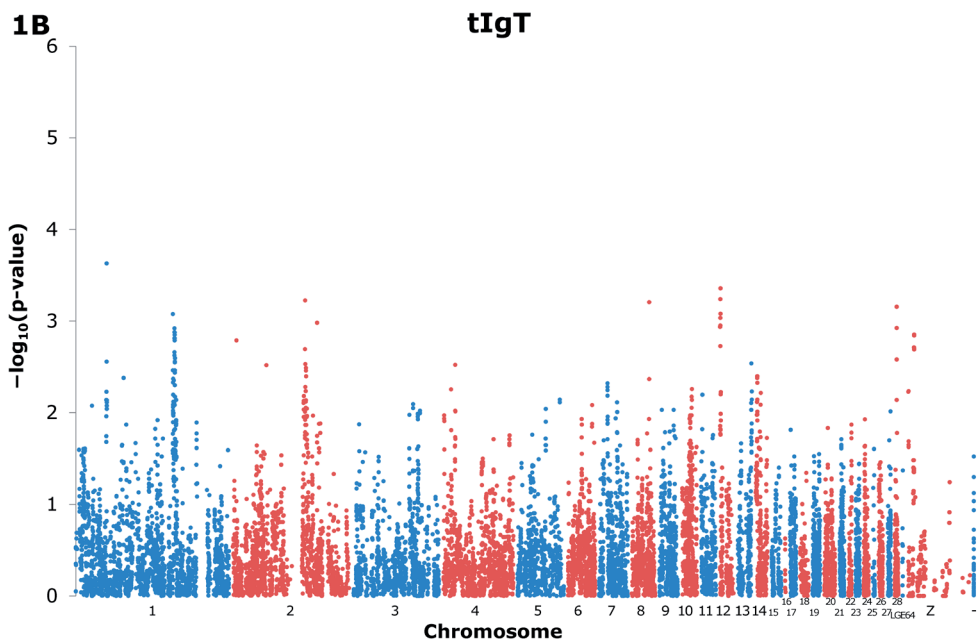
[c] The reported NAb h^2 and m^2 are of a previous study (Berghof et al., 2015).

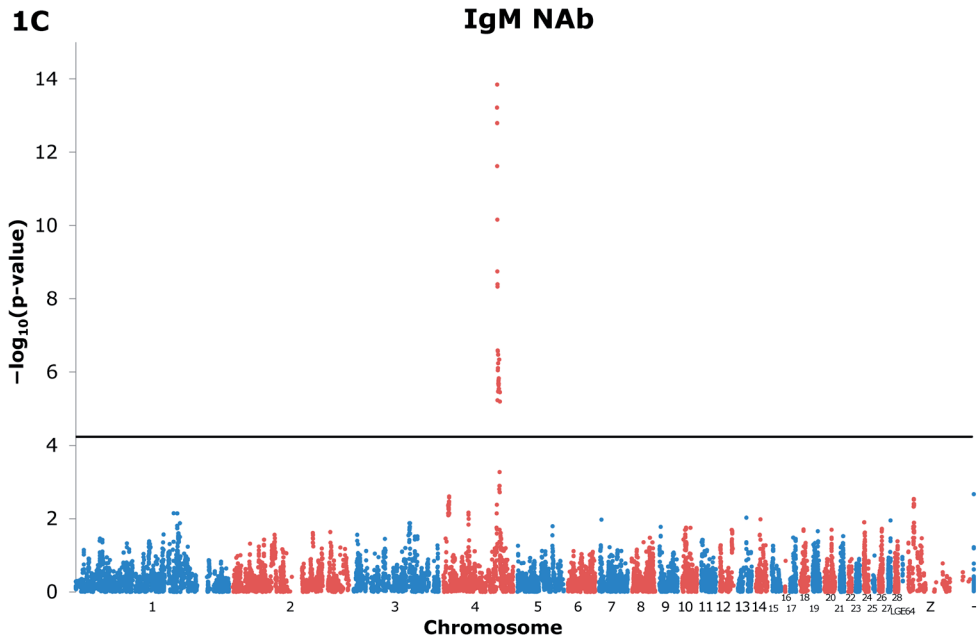
[d] The h^2 and m^2 are estimated based on a $\log_{10}$-tranformation of the data.

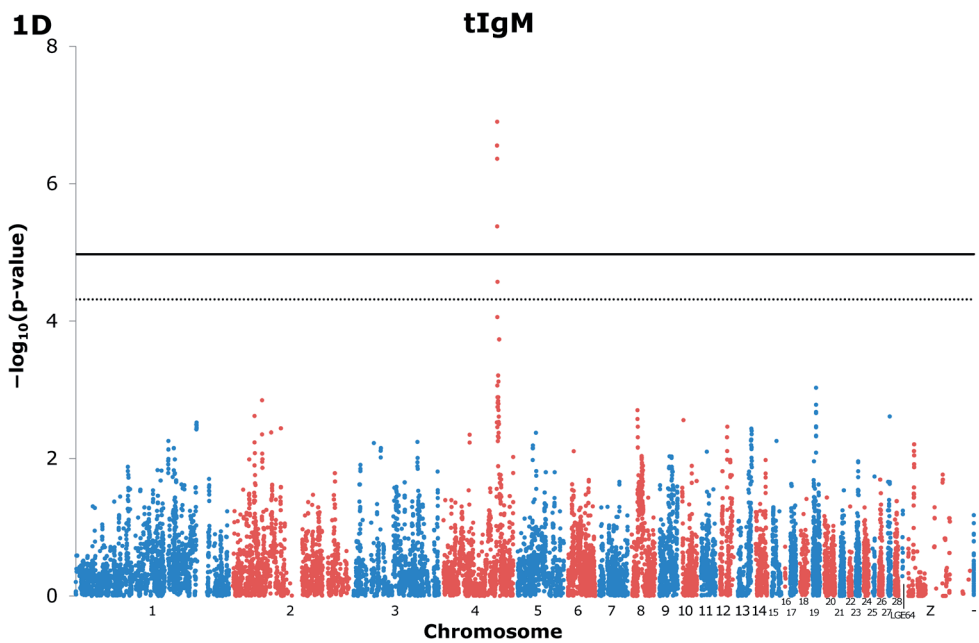
IgA NAb/tIgA, and 0.08 for IgG NAb/tIgG. In addition, genetic and phenotypic correlations between all total antibody concentrations were estimated (see Supplementary Table 2).

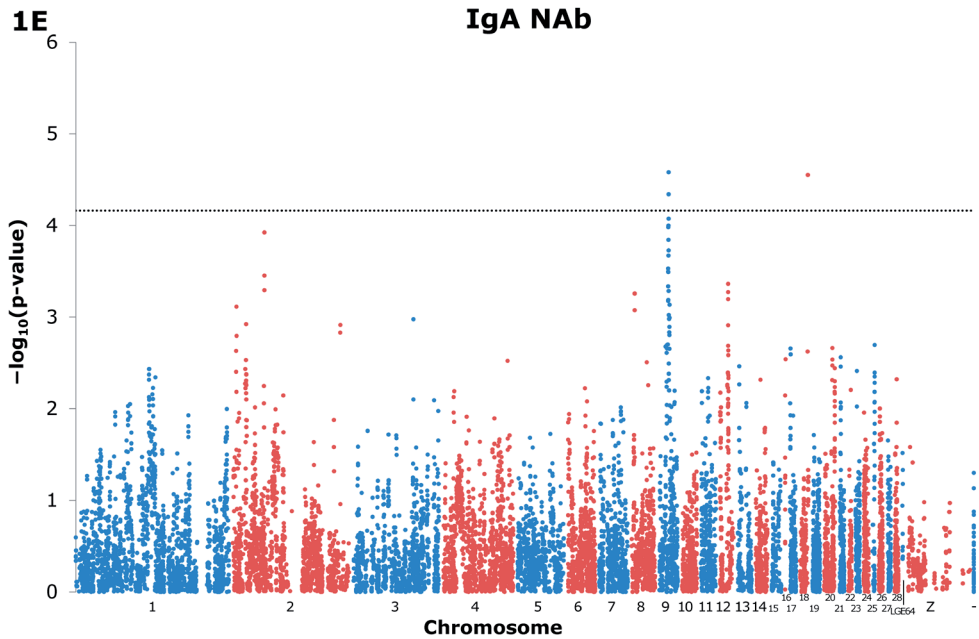
Quality control of the SNP resulted in removal of 37,053 SNP, because they were monomorphic (step1), and an additional removal of 5,005 SNP, because of a low number of observations per genotype class or a strong deviation from Hardy-Weinberg equilibrium (step 2-4) (see Supplementary Table 1). In total, 15,579 SNP were used for the GWAS, though the actual number of used SNP per trait varied between 15,431 and 15,578 SNP due to missing phenotypes. Figure 1 shows the 'Manhattan plots' of the GWAS. Results shown for IgM NAb and all total antibody concentrations (tIgT, tIgM, tIgA, and tIgG) were adjusted for inflation factor λ, which was estimated to be 1.67 for IgM NAb, 1.14 for tIgT, 1.23 for tIgM, 1.27 for tIgA, and 1.13 for tIgG. This indicates a deviation of the p-values from their expected distribution under the null hypotheses of no genetic association. After correcting the test statistics and subsequently recalculating the p-values, all new λ were below 1. Inflation factors for IgT NAb, IgA NAb, and IgG NAb were below 1.1, and required no adjustment. For IgT NAb, and IgA NAb, none of the SNP were significantly associated (FDR ≤ 0.05), but some regions showed suggestive associations (FDR ≤ 0.20) (see Supplementary Table 3). For IgM NAb, and tIgM several SNP were significantly associated (see Supplementary Table 3). For IgG NAb, tIgT, tIgA, and tIgG no significant or suggestive associations were detected.

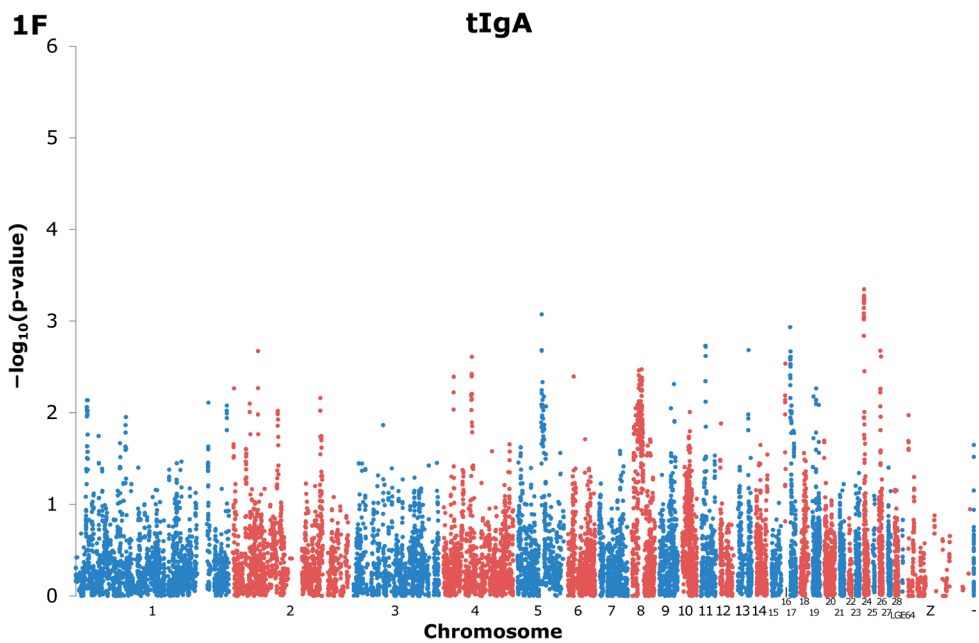

1A
IgT NAb
−log10(p-value)
Chromosome

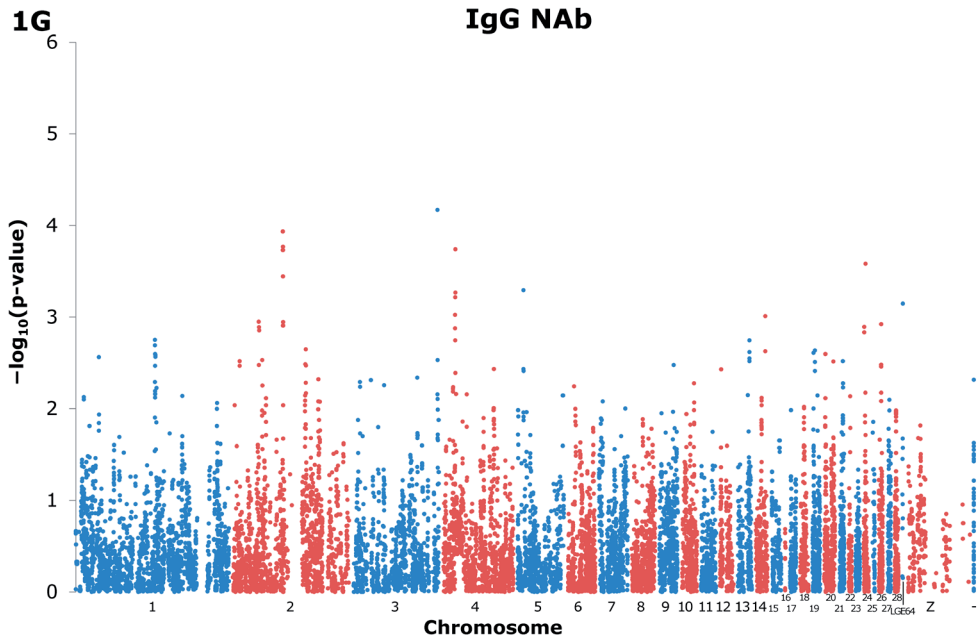

1B
tIgT
−log10(p-value)
Chromosome


1C
IgM NAb
−log10(p-value)
14
12
10
8
6
4
2
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 LGE64 Z
Chromosome


1D
tIgM
−log10(p-value)
8
6
4
2
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 LGE64 Z
Chromosome


1E
IgA NAb
−log10(p-value)
Chromosome


1F
tIgA
−log10(p-value)
Chromosome


1G
IgG NAb
−log10(p-value)
0
1
2
3
4
5
6
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 LGE64 Z
Chromosome

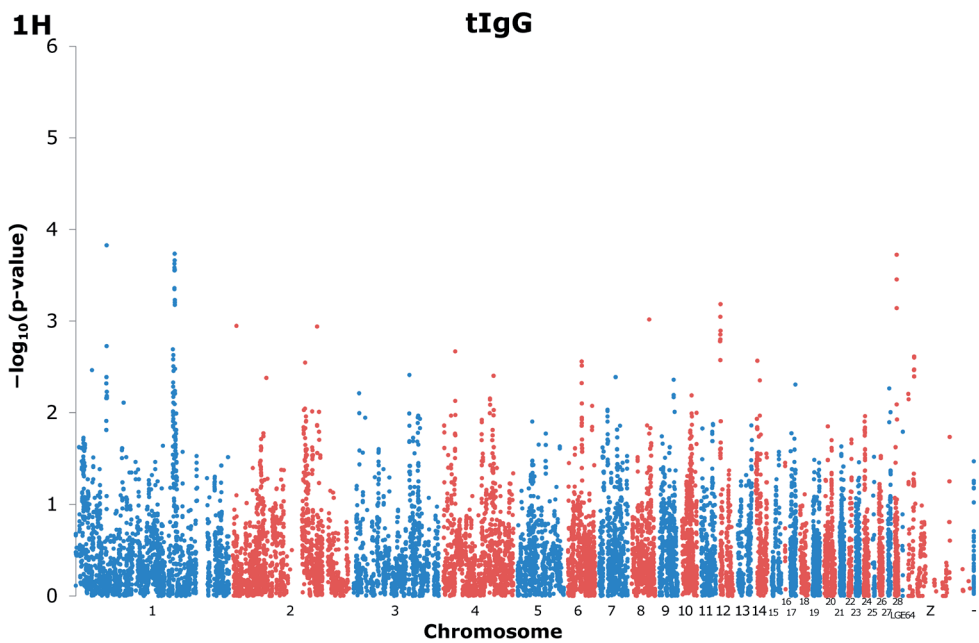

1H
tIgG
−log10(p-value)
0
1
2
3
4
5
6
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 LGE64 Z
Chromosome

Figure 1. Manhattan plots of genome-wide association studies for KLH-binding natural antibody (NAb) titers and total antibody concentrations (µg/ml) in a White Leghorn chicken population around 16 weeks of age. The figures show: A: total KLH-binding NAb (IgT) titer; B: total antibody (tIgT) concentration; C: KLH-binding IgM NAb (IgM) titer; D: total IgM (tIgM) concentration; E: KLH-binding IgA NAb (IgA) titer; F: total IgA (tIgA) concentration; G: KLH-binding IgG NAb (IgG) titer; H: total IgG (tIgG) concentration. tIgT, tIgM, tIgA, and tIgG were $\log_{10}$-tranformed for the analyses. Genomic control was applied to the test statistic for tIgT, IgM NAb, tIgM, tIgA, and tIgG. On the x-axis is the physical position of the 15,579 used SNP across the chicken genome (chromosomes 1-28, linkage groups LGE64, chromosome Z, and unplaced represented by -). Positions are based on Gallus_gallus-5.0. On the y-axis is the $-\log_{10}(\text{p-value}_{SNP})$ for association of the SNP with the investigated trait. Note the different y-axes for IgM and tIgM. The false discovery rate (FDR) threshold was set at 0.05 for significant SNP (solid horizontal line) and at 0.20 for suggestive SNP (dashed horizontal line). If no SNP reached the FDR threshold, the threshold could not be estimated. For IgM no $-\log_{10}(\text{p-value}_{SNP})$ reached $0.05 \leq FDR \leq 0.20$, and therefore $FDR \lesssim 0.20$ is not shown.

Table 3 and Table 4 characterize the significantly and suggestively associated genomic regions based on the GWAS results. The associated regions and additional information can be found in Table 3. The lead SNP and additional information can be found in Table 4. Supplementary Table 3 gives an overview of associated genotyped and imputed SNP.

One genomic region showed an association with IgT NAb and tIgM, and the strongest association was for IgM NAb. The genomic region was located on chromosome 4 around 70 M base pair (bp), and consisted of 3 suggestive SNP for IgT NAb, 4 significant and 1 suggestive SNP for tIgM, and 35 significant SNP for IgM NAb. The genomic region on chromosome 4 had its lead SNP for IgT NAb on 69,587,709 bp (rs313004783), for tIgM on 69,814,286 bp (rs313437715), for IgM NAb on 69,702,481 bp (rs15614874). Remarkably the heterozygous genotype class was not significantly different from one of the homozygous genotype classes, indicating full dominance. The lead SNP variance as a percentage of the additive genetic variance was approximately 16.0% for IgT NAb, 14.1% for tIgM, and 57.6% for IgM NAb, illustrating that the genomic region on chromosome 4 has a major effect on the genetic variation of IgT NAb, tIgM, and especially on IgM NAb.

The first of the two genomic regions detected for IgA NAb was located on chromosome 9

Table 2. Estimated genetic correlations and phenotypic correlations of KLH-binding natural antibody (NAb) titers and total antibody concentrations (µg/ml) in a White Leghorn chicken population around 16 weeks of age. The table shows the correlations of: total KLH-binding NAb (IgT) titer and total antibody (tIgT) concentration, KLH-binding IgM NAb (IgM) titer and total IgM (tIgM) concentration, KLH-binding IgA NAb (IgA) titer and total IgA (tIgA) concentration, and KLH-binding IgG NAb (IgG) titer and total IgG (tIgG) concentration. tIgT, tIgM, tIgA, and tIgG were $\log_{10}$-tranformed for the analyses. SE are shown in parentheses.

	Genetic correlation	Phenotypic correlation
IgT / tIgT[a]	−0.27 (0.31)	0.03 (0.03)
IgM[b] / tIgM	0.91 (0.06)	0.41 (0.02)
IgA / tIgA	0.38 (0.22)	0.26 (0.03)
IgG / tIgG	−0.61 (0.55)	0.08 (0.03)

[a] tIgT = tIgM + tIgA + tIgG

[b] Dam component was fixed at boundary (0) in ASReml analysis.

Table 3. Genomic regions significantly or suggestively associated with KLH-binding natural antibody (NAb) titers and total antibody concentrations (µg/ml) in a White Leghorn chicken population around 16 weeks of age. Analyzed traits are: total KLH-binding NAb (IgT) titer, KLH-binding IgM NAb (IgM) titer, KLH-binding IgA NAb (IgA) titer, KLH-binding IgG NAb (IgG) titer, total antibody (tIgT) concentration, total IgM (tIgM) concentration, total IgA (tIgA) concentration, and total IgG (tIgG) concentration. tIgT, tIgM, tIgA, and tIgG were log_{10}-tranformed for the analyses. The table shows: trait, chromosome of associated genomic region, base pair (bp) start - bp stop, number of significant (False Discovery Rate (FDR) ≤ 0.05) SNP associated, and number of suggestive (0.05 ≤ FDR ≤ 0.20) SNP associated. Positions are based on Gallus_gallus-5.0.

					# SNP	
Trait	**Chromosome**	**Start (bp)**	**-**	**Stop (bp)**	**Significant**	**Suggestive**
IgT	4	69,587,709	-	69,814,286	0	3
IgM		69,587,709	-	73,362,701	35	0
tIgM		69,587,709	-	70,109,615	4	1
IgA	9	12,230,193	-	12,261,658	0	2
IgA	18	9,984,590	-	10,154,530	0	3

around 12 Mbp, and consisted of 2 suggestive SNP. The lead SNP was located at 12,261,658 bp (rs15969591). The lead SNP variance was approximately 13.5% of the additive genetic variance for IgA NAb. The second genomic region for IgA NAb was located on chromosome 18 around 10 Mbp. This region consists of 3 suggestive neighboring SNP with equal p-values. The region showed complete dominance. The variance of the region was approximately 0.2% of the additive genetic variance for IgA NAb.

The significantly associated region on chromosome 4 was imputed to whole genome sequence. The region selected for further investigation ranged from approximately 64.7 Mbp to 74.8 Mbp. After quality control 43,675 SNP remained for IgM NAb, and 39,792 SNP for tIgM, deviating because of missing phenotypes for tIgM. Figure 2 shows the results of the association study for IgM NAb and tIgM of the selected region on chromosome 4. The highest signal for IgM NAb and tIgM was detected in the region 69.5 Mbp to 70.2 Mbp with 3,153 SNP (see Figure 2C). Candidate genes (5'-to-3' direction) in this region are: PDS5 cohesin associated factor A (*PDS5A*), ubiquitin conjugating enzyme E2 K (*UBE2K*), small integral membrane protein 14 (*SMIM14*), UDP-glucose 6-dehydrogenase (*UGDH*), lipoic acid synthetase (*LIAS*), ribosomal protein L9 (*RPL9*), klotho beta (*KLB*), WD repeat domain 19 (*WDR19*), replication factor C subunit 1 (*RFC1*), kelch like family member 5 (*KLHL5*), transmembrane protein 156 (*TMEM156*), family with sequence similarity 114 member A1 (*FAM114A1*), toll-like receptor 1 family member B (*TLR1B*), toll-like receptor 1 family member A (*TLR1A*), Kruppel like factor 3 (*KLF3*), and TBC1 domain family member 1 (*TBC1D1*) (from NCBI (NCBI Resource Coordinators, 2016) and Ensembl (Release 87) (Yates et al., 2015)) (see Figure 2C). The strongest associated subregion contained *FAM114A1*, *TLR1B*, *TLR1A*, and *KLF3*.

The 3,153 SNP in this region were checked for amino acid substitutions in coding regions, and consequently changes in protein functioning as a result of the nucleotide change. The Ensembl Variant Effect Predictor predicted 28 SNP to result in different amino acids. All

Table 4. Lead SNP (SNP with highest p-value) within genomic regions significantly or suggestively associated with KLH-binding natural antibody (NAb) titers and total antibody concentrations (µg/ml) in a White Leghorn chicken population around 16 weeks of age. Analyzed traits are: total KLH-binding NAb (IgT) titer, KLH-binding IgM NAb (IgM) titer, KLH-binding IgA NAb (IgA) titer, KLH-binding IgG NAb (IgG) titer, total antibody (tIgT) concentration, total IgM (tIgM) concentration, total IgA (tIgA) concentration, and total IgG (tIgG) concentration. tIgT, tIgM, tIgA, and tIgG were $\log_{10}$-tranformed for the analyses. The table shows: trait, chromosome of associated genomic region, position of lead SNP, name of lead SNP, $-\log_{10}(\text{p-value}_{\text{lead SNP}})$ (*italic* for suggestive association), number of chickens per genotype class, effect of genotype classes and SE in titer points for NAb or µg/ml for total antibody concentrations, and percentage of additive genetic variance explained by the lead SNP ($\sigma^2_{SNP}/\sigma^2_a$ * 100%). Genotype classes with significantly (p-value ≤ 0.05) different effects are indicated by different superscripts (ˣ, ʸ, or ᶻ). Positions are based on Gallus_gallus-5.0.

Trait	Chromosome	Position	SNP name	$-\log_{10}(p)$	n_{AA}	n_{AB}	n_{BB}	AA (SE)[a]	AB[a]	BB (SE)[a]	$\sigma^2_{SNP}/\sigma^2_a$
IgT	4	69,587,709	rs313004783	5.21	480	807	338	−0.34ʸ (0.08)	0ᶻ	0.09ʸᶻ (0.08)	16.0%
IgM		69,702,481	rs15614874	13.84[b]	477	824	326	−0.58ʸ (0.06)	0ᶻ	0.01ᶻ (0.06)	57.6%
tIgM		69,814,286	rs313437715	6.90[b]	465	821	333	−40.7ʸ [c]	0ᶻ	6.2ᶻ [c]	14.1%
IgA	9	12,261,658	rs15969591	4.58	814	708	86	0.18 (0.06)	0	0.53 (0.13)	13.5%
IgA[d]	18	10,131,465	rs10731438	4.55	1,133	430	45	−0.05ʸ (0.07)	0ʸ	0.78ᶻ (0.18)	0.2%

[a] Genotype effects are compared to the AB genotype class, which was set to 0 (zero).

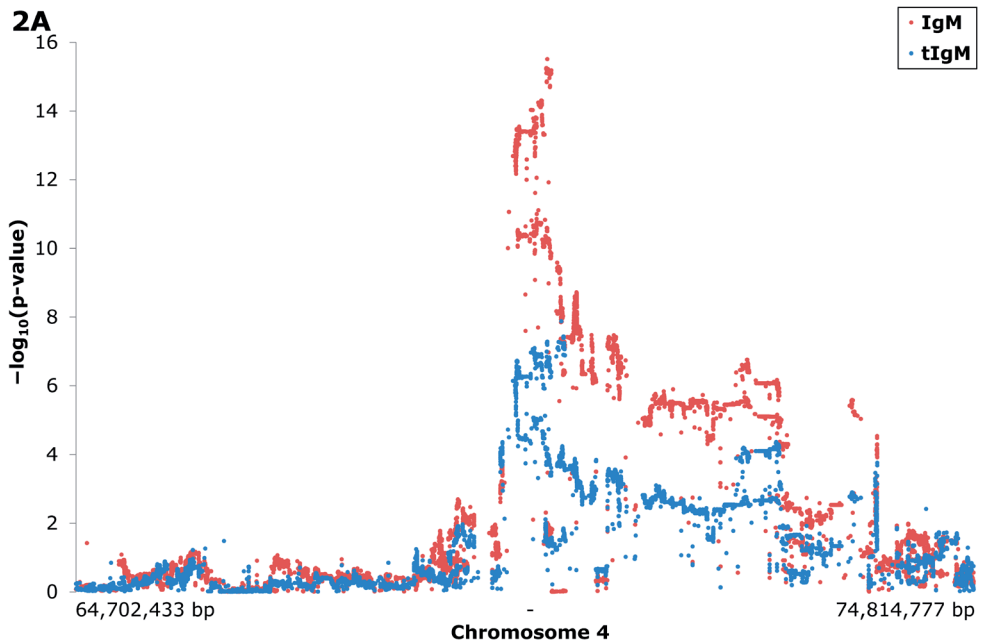
[b] Genomic control was applied to the test statistic.

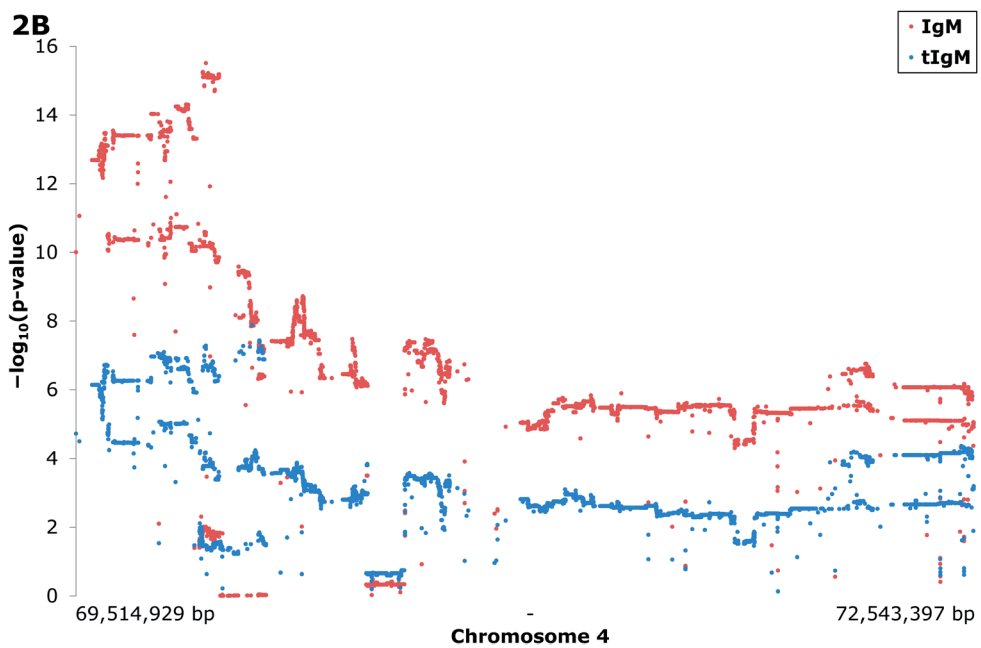
[c] The estimated effects are reported in µg/ml, but were calculated from the predicted values on a $\log_{10}$-scale. Predicted values were 2.43 for AA, 2.49 for AB, and 2.50 for BB. Estimated effects based on $\log_{10}$-analysis were −0.06 (0.01) for AA, and 0.01 (0.01) for BB.

[d] Region contains 3 SNP with similar $-\log_{10}(\text{p-value}_{SNP})$. The genotyped SNP is reported.

28 SNP could be predicted with either SIFT or PolyPhen-2 or both. SIFT predicted amino acid substitutions to be "tolerated" for 11 SNP, and "deleterious" for 2 SNP. The remaining 15 SNP could not be predicted. PolyPhen-2 predicted amino acid substitution to be "benign" for 22 SNP, "possibly damaging" for 1 SNP, and "probably damaging" for 1 SNP. SIFT and PolyPhen-2 gave both the maximum impact score ("deleterious", and "probably damaging") to 1 SNP (69,965,939 bp), which is considered the most likely candidate causal variant for the major effects on IgT NAb, tIgM, and especially on IgM NAb. This SNP consist of a cytosine/guanine polymorphism, and results in a phenylalanine (F)/leucine (L) amino acid substitution in *TLR1A* at protein position 126. The C-variant had an allele frequency of 0.45, and the G-variant had an allele frequency of 0.55. Based on Figure 1 from Keestra et al. (2007), the SNP is located in leucine-rich repeat (LRR)4.

Significance and effects of the predicted causal variant on all measured traits can be found in Table 5. IgT NAb, IgM NAb, IgG NAb, and tIgM showed a significant effect of the predicted causal variant. For these traits (except IgG NAb), the GG genotype class had significantly lower phenotypic values than the


2A
IgM
tIgM
16
14
12
10
8
6
4
2
0
−log10(p-value)
64,702,433 bp
-
74,814,777 bp
Chromosome 4


2B
IgM
tIgM
16
14
12
10
8
6
4
2
0
−log10(p-value)
69,514,929 bp
-
72,543,397 bp
Chromosome 4

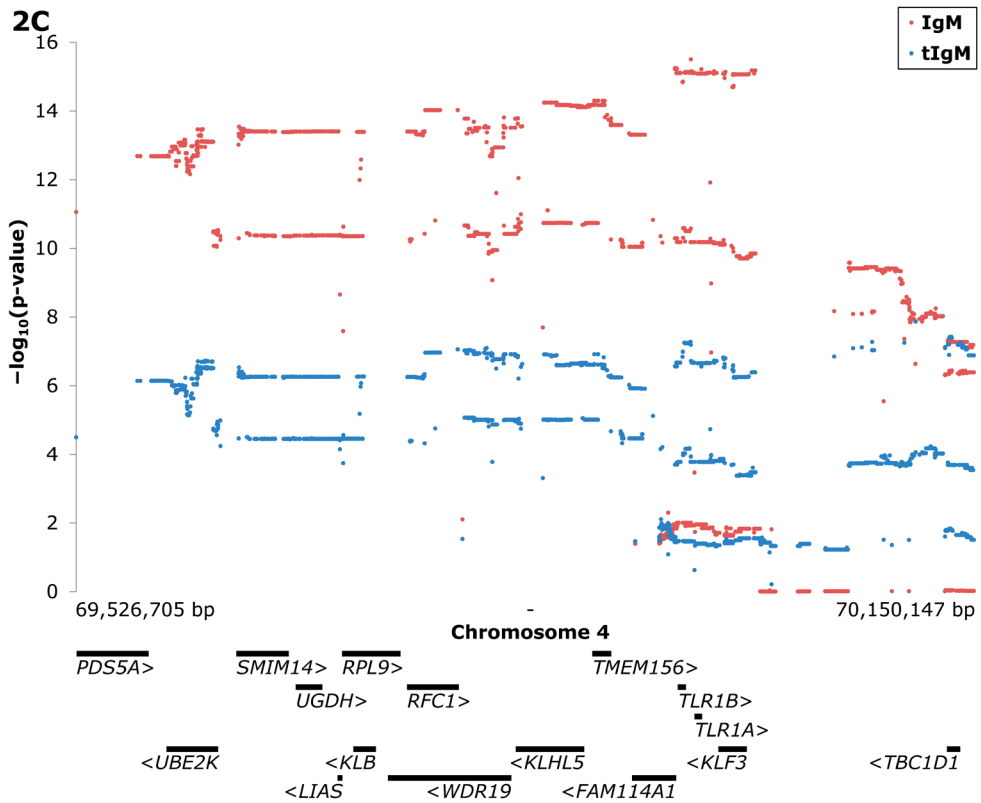


Figure 2. Manhattan plots of association studies of the selected region on chromosome 4 for KLH-binding IgM NAb (IgM) titer in red, and total IgM (tIgM) concentration (log_{10}-tranformed for the analyses) in blue in a White Leghorn chicken population around 16 weeks of age. On the y-axes is the $-log_{10}(p\text{-value}_{SNP})$ for the association of the investigated SNP with IgM and tIgM after genomic control was applied. A: On the x-axis is the physical position of the approximately 40,000 SNP used on chromosome 4 for the selected region; B: Zoom of peak-region in figure A ($-log_{10}(p\text{-value}_{SNP}) \geq 5$); C: Zoom of peak-region in figure B ($-log_{10}(p\text{-value}_{SNP}) \geq 10$ for IgM, and $-log_{10}(p\text{-value}_{SNP}) \geq 6$ for tIgM), including candidate gene overview (Yates et al., 2015). Positions are based on Gallus_gallus-5.0. Arrows ("<" and ">") indicate direction of the genes. Abbreviations genes: *PDS5A*: PDS5 cohesin associated factor A; *UBE2K*: ubiquitin conjugating enzyme E2 K; *SMIM14*: small integral membrane protein 14; *UGDH*: UDP-glucose 6-dehydrogenase; *LIAS*: lipoic acid synthetase; *RPL9*: ribosomal protein L9; *KLB*: klotho beta; *WDR19*: WD repeat domain 19; *RFC1*: replication factor C subunit 1; *KLHL5*: kelch like family member 5; *TMEM156*: transmembrane protein 156; *FAM114A1*: family with sequence similarity 114 member A1; *TLR1B*: toll-like receptor 1 family member B; *TLR1A*: toll-like receptor 1 family member A; *KLF3*: Kruppel like factor 3; *TBC1D1*: TBC1 domain family member 1 (from NCBI (NCBI Resource Coordinators, 2016) and Ensembl (Release 87) (Yates et al., 2015)).

CG and the CC genotype classes, which were not significantly different from each other (full dominance). The estimated genetic variance explained by the predicted causal variant was between 7.3% and 15.8% for IgT NAb, IgG NAb, and tIgM, and was 63.5% for IgM NAb. IgA NAb, tIgT, tIgA, and tIgG were not significantly associated.

Table 5. Significance and effects of the predicted causal variant (SNP on chromosome 4 position 69,965,939 bp, based on Gallus_gallus-5.0) of KLH-binding IgM natural antibody (NAb) (IgM) titer and total IgM (tIgM) concentration (µg/ml) on all measured KLH-binding NAb titers and total antibody concentrations (µg/ml) in a White Leghorn chicken population around 16 weeks of age. Analyzed traits are: total KLH-binding NAb (IgT) titer, KLH-binding IgM NAb (IgM) titer, KLH-binding IgA NAb (IgA) titer, KLH-binding IgG NAb (IgG) titer, total antibody (tIgT) concentration, total IgM (tIgM) concentration, total IgA (tIgA) concentration, and total IgG (tIgG) concentration. tIgT, tIgM, tIgA, and tIgG were log_{10}-tranformed for the analyses. Only significant SNP effects are reported. The estimated genotype effects for tIgT, tIgM, tIgA, and tIgG are reported in µg/ml. The table shows: trait, p-value, effect of genotype classes (and SE) in titer points for NAb or µg/ml for total antibody concentrations, and percentage of additive genetic variance explained by the investigated SNP ($\sigma^2_{SNP}/\sigma^2_a * 100\%$). Genotype classes with significantly (p-value ≤ 0.05) different effects are indicated by different superscripts ([y], or [z]).

Trait	p-value	GG (SE)[ab]	CG[ab]	CC (SE)[ab]	$\sigma^2_{SNP}/\sigma^2_a$
IgT	<0.001	−0.35[y] (0.08)	0[z]	0.08[z] (0.08)	15.8%
IgM	<0.001	−0.61[y] (0.06)	0[z]	0.04[z] (0.06)	63.5%
IgA	0.45				
IgG	0.05	−0.11 (0.08)	0	0.15 (0.09)	7.3%
tIgT	0.70				
tIgM	<0.001	−39.31[y c]	0[z]	6.64[z c]	13.3%
tIgA	0.84				
tIgG	0.26				

[a] Genotype effects are compared to the CG genotype class, which was set to 0 (zero).

[b] The number of observation per genotype class varied between 457 and 475 for GG, 803 and 831 for CG, and 313 and 321 for CC.

[c] The estimated effects are reported in µg/ml, but were calculated from the predicted values on a log_{10}-scale. Predicted values were 2.43 for GG, 2.49 for CG, and 2.50 for CC. Estimated effects based on log_{10}-analysis were −0.06 (0.01) for GG, and 0.01 (0.01) for CC.

Discussion

This study was designed to identify genomic regions influencing variation in KLH-binding NAb titers in layer chickens. A strong association was located on chromosome 4 near *TLR1A* and *TLR1B*, which suggested an essential and fundamental role of TLR in NAb levels. We hypothesized that the detected polymorphism near TLR could also influence total antibody concentrations. Therefore, we additionally analyzed the plasma samples for total antibody concentrations.

Relatively few GWAS have focused on detecting genomic regions associated with innate and adaptive immunity in chicken (Cheng et al., 2013; Schmid et al., 2015; Zhang et al., 2015). Though two KLH-binding NAb association studies with dedicated SNP sets were conducted across chicken lines (Biscarini et al., 2010; Sun et al., 2013a). To the best of our knowledge, this study is the first genome-wide association study on KLH-binding NAb levels in chickens. None of previously associated genes could be confirmed in this study, likely because of the high genetic uniformity in our data set as a result of a within line study instead of an across line study. No other study found the associated region on chromosome 4 reported in this study, which might indicate that this association is unique for this chicken line. This is also

the first study that investigated heritabilities of total antibody concentrations or performed GWAS on all total antibody isotype concentrations in chickens.

57,636 SNP were used to perform the GWAS. 73.0% of the SNP were non-informative, mainly because the SNP were fixed (62.2%). This is in agreement with previously reported genetic uniformity in White Leghorn layer chicken lines (Megens et al., 2009). In total 15,580 SNP passed quality control. For 5 out of 8 analyzed traits population stratification was found to be present, which suggests family structures within the studied population that are not captured by the pedigree information. Other factors, like heterogeneous variance or model misspecification, likely also contributed to this, since inflation factors were not of equal size among the traits. Especially for IgM NAb, the population stratification was high. After correcting the test statistics for the inflation factor λ, the new inflation factor indicated that IgM NAb was overcorrected ($\lambda = 0.88$). Thereby being possibly too conservative on the associations with IgM NAb.

Heritabilities of tIgT and tIgG antibody concentrations were low, but comparable to heritabilities of IgT and IgG NAb titers (Berghof et al., 2015). This indicates a strong environmental influence on IgG antibodies and consequently on IgT, since IgT mainly consists of IgG antibodies: all genetic and phenotypic correlations were higher than 0.8 (this study, and Berghof et al. (2015)). However IgT NAb, but not tIgT, was suggestively associated to the same region of IgM NAb and tIgM. This confirms the high genetic correlation of IgT NAb/IgM NAb (Berghof et al., 2015), and the moderate genetic correlation of tIgT/tIgM (this study).

The heritability of tIgA was twice the heritability of IgA NAb. Interestingly, similar concentrations for tIgA and tIgM were found, which is the first time to be reported. Genetic and phenotypic correlations between IgA NAb and tIgA were positive, and low to moderate. This suggests that variation in IgA NAb and tIgA are mainly regulated by different mechanisms. The role of blood IgA antibodies is not well understood so far. It has been suggested to be a response isotype with a pro-inflammatory function, similar to IgG antibodies (Van Egmond et al., 2001; Cerutti, 2008; Bekeredjian-Ding et al., 2009). However, in chicken most of the intestinal IgA is secreted via the bile (Lebacq-Verheyden et al., 1974; Rombout et al., 1992), which means that intestinal IgA first has to be transported through the blood. IgA in blood might therefore represent the intestinal health status in chickens.

Two suggestively associated regions were identified for IgA NAb. These regions were not confirmed by tIgA concentration, even though a positive genetic correlation was estimated. This is the first time an association study on IgA (either NAb or total antibody concentration) in healthy individuals has been done. Observed results on IgA NAb were suggestive, and therefore these results first need to be confirmed in independent studies.

The heritability of tIgM was low, but twice the heritability of IgM NAb (Berghof et al., 2015). IgM NAb have been found to be influenced by maternal environmental effects (Berghof et al., 2015). In case maternal effects are not accounted for in genetic analyses, heritabilities will be overestimated as was seen for IgM NAb (Berghof et al., 2015). The size of the present

data set did not allow detection of maternal environmental effect for IgM NAb. The estimated heritability (without maternal environmental effect) for IgM NAb in this study population was 0.31 (data not shown), which is similar to the heritability of tIgM. In addition, the genetic correlation of IgM/tIgM was very high. This suggests that maternal environmental effects for tIgM are present, but could not be estimated, and resulted in overestimation of tIgM heritability.

One significant genomic region was identified for IgM NAb and tIgM on chromosome 4 around 70 Mbp. When correcting the IgM NAb and tIgM data for the estimated effects, the genomic region disappeared (data not shown), which shows that only one genomic region (i.e. one gene with one causal variant) is responsible for the observed effects.

All of the candidate genes in the IgM associated region on chromosome 4 have been related to organism development, cell cycle, cell proliferation, metabolism, cancer development, viral infections, or a combination (mostly based on homologues in human and mouse studies), except for *SMIM14*, *TMEM156*, *FAM114A1* (no literature available) (NCBI Resource Coordinators, 2016). This implies that all candidate genes have a potential to influence IgM antibodies in some way. However, some genes are reported in association with antibodies or B cells, which makes them more likely candidates: *KLF3* knockout mice were reported to have significantly impaired B cell differentiation, including NAb-producing B1 cells (Vu et al., 2011). TLR, including TLR1/TLR6 (homologue/paralogue to TLR1A/TLR1B (Huang et al., 2011)), are well-known for their essential role in antibody production after stimulation of B cells, including B1 cells (Ray et al., 2005; Alugupalli et al., 2007; Bekeredjian-Ding et al., 2009). TBC1D1 has been reported to have a potential downstream role of silencing of pre-BCR signaling in acute lymphoblastic leukemia in human (Bicocca et al., 2012). However, none of these studies reported an effect on IgM NAb or tIgM without deliberate stimulation of B cells in healthy individuals, as is the case in this study.

Fine mapping of the associated region allowed to predict changes in protein functioning as a result of the nucleotide changes in coding regions. A nucleotide change within *TLR1A* was predicted to have severe impact on protein folding/functioning, making this SNP the most likely candidate for the major effects on IgT NAb, tIgM, and especially on IgM NAb. TLR1A, also known as TLR1-like a (TLR1La), TLR1.1, TLR1/6/10, and TLR16 (Temperley et al., 2008), is one of the 10 known chicken TLR (see Brownlie et al. (2011) for a review on avian TLR). TLR are among the most studied immune receptors. They are a family of transmembrane proteins that recognize conserved molecular patterns (pathogen-/microbe-associated molecular patterns; PAMP/MAMP), and are conserved in evolution (Leulier et al., 2008). TLR1A dimerizes with TLR2 (either TLR2A or TLR2B), and recognizes peptidoglycans and related structures, including FSL-1, and PAM_3CSK_4 (Keestra et al., 2007; Higuchi et al., 2008), though some anomalies exist between studies (Brownlie et al., 2011). TLR1A contains 19 leucine-rich repeats (LRR) (Keestra et al., 2007), of which LRR6-16 are required for ligand specificity (Keestra et al., 2007), and region close to the C-terminal end for dimerization with TLR2 in humans (Gautam et al., 2006; Jin et al., 2007). The candidate

SNP is located in LRR4 near the N-terminal end, suggesting not a direct influence on ligand recognition. Instead, it is likely influencing the tertiary protein structure, or mediation of coreceptors with TLR (see Van Bergenhenegouwen et al. (2013) for a review), or both. Also, only coding regions were checked for amino acid substitutions, but not f.e. promotor regions influencing expression of genes. Based on the data in this study, no conclusion can be drawn on whether the most likely causal variant results in a complete loss of function or in reduced functioning. Nevertheless, full dominance was observed for this *TLR1A* variant, suggesting that one functional copy of the gene results in a sufficient expression of functional TLR1A heterodimer and enhancement by the coreceptors for activation. Future studies should confirm the predicted *TLR1A* variant, and its functionality.

The most likely causal variant has an effect on IgM NAb, and tIgM. We hypothesize a role of this *TLR1A* variant on naive B cell maturation, proliferation, survival, IgM production, or a combination of these. The most likely causal variant can affect the three stages of the (avian) B cell development: pre-bursal (only before hatch), bursal, and post-bursal (Ratcliffe et al., 2014). Considering *TLR1A* as the most likely candidate, the effect on B cells and antibody levels is expected after exposure to PAMP, i.e. after hatch. *TLR1A* is expressed on various types of cells, including macrophages and B cells, and in various organs, including thymus, spleen, liver, and bursa of Fabricius (Iqbal et al., 2005; Brownlie et al., 2011). The effect of the *TLR1A* variant on IgM levels can therefore be either directly on B cells, or indirectly on B cells via for example macrophages, or both. An effect of the *TLR1A* variant is also expected on other cells of the immune system, both of the innate and adaptive part. This *TLR1A* variant is of particular interest, because it might explain part of the association of especially IgM NAb with survival in chicken (Sun et al., 2011; Wondmeneh et al., 2015). Yilmaz et al. (2005) suggested that the TLR1 and TLR2 subfamilies are under positive selection (favoring polymorphisms) in chicken, while all other TLR are under negative/purifying selection (favoring conserved structures) (Yilmaz et al., 2005). Huang et al. (2011) suggested that only certain sites of TLR1 might be under positive selection, but that TLR1 is mostly under purifying selection (Huang et al., 2011). Nevertheless, the results imply that *TLR1A* is not essential for healthy chickens, but that it does influence IgM levels.

The identified region on chromosome 4 has previously been associated with many other characteristics in chickens (layer, broiler, or both) (based on Animal QTLdb, (Hu et al., 2016b)). Only two of these are directly related to health and disease susceptibility: The same region was found for natural autoantibodies IgM levels (Bao, submitted), and for susceptibility to Marek's disease virus (Heifetz et al., 2007). Though this association with Marek's susceptibility was stronger around TLR3 (also located on chromosome 4, around 62 Mbp), which is involved in the recognition of viruses, including Marek's disease virus infections (Hu et al., 2016a). Other characteristics associated to these region were for example age at first egg (Podisi et al., 2011), eggshell breaking strength (Sasaki et al., 2004), eggshell color (Goto et al., 2011; Wolc et al., 2014), egg weight (Tuiskula-Haavisto et al., 2002; Sasaki et al., 2004; Schreiweis et al., 2006), feed intake (Tuiskula-Haavisto et al., 2002), growth/body weight at several ages (Carlborg et al., 2004; Zhou et al., 2006a; Nadaf

et al., 2009; Ankra-Badu et al., 2010; Podisi et al., 2011; Goraga et al., 2012; Podisi et al., 2013; Nassar et al., 2015), liver weight (Navarro et al., 2005), and spleen weight (Zhou et al., 2006b). To investigate these associations, production traits of 222 females of the study population were previously collected (described in Van der Klein et al. (2015)), and could therefore be tested for their association with the most likely causal variant. No significant effect of the *TLR1A* variant was observed on number of laid eggs from 25 weeks of age onwards, eggshell breaking strength, eggshell whiteness (related to eggshell color), egg weight, feed conversion ratio, body weight, and survival day. Only the number of laid eggs between 17 and 24 weeks of age tended ($p = 0.09$) to a significant association with the *TLR1A* variant, where the genotype associated with lower IgM levels was associated with higher egg production (data not shown). Such a beneficial effect of the *TLR1A* variant on one of the most important production traits in layer chickens and the high sanitary barriers on the breeding nucleus with low pathogenic pressure might explain why the variant is present in the study population, even though it might impair (humoral) immunity.

Remarkably the genetic correlations of KLH-binding IgT NAb/tIgT, and KLH-binding IgG NAb/tIgG were negative. This suggest that KLH-binding IgT NAb and tIgT, and KLH-binding IgG NAb and tIgG are genetically regulated in a similar way, but with opposing effects. However, phenotypically there were very weak correlations of KLH-binding IgT NAb/tIgT and KLH-binding IgG NAb/tIgG. In addition KLH-binding IgT NAb and KLH-binding IgG NAb titers were significantly associated with the *TLR1A* variant, but tIgT and tIgG concentrations were not. This suggests that a genetic predisposition for low (or high) levels of KLH-binding IgT and KLH-binding IgG NAb or different genotypes for the *TLR1A* variant do not negatively (or positively) affect the total IgT and IgG antibody concentrations, implying that the humoral adaptive immune response and memory formation are not negatively influenced by low NAb levels or the *TLR1A* variant.

In this study, we show the potential of using a large database with defined immunological traits to discover underlying genomic regions and new fundamental components in the development and functioning of the immune system. Recently in humans, similar approaches have been used to detect genetic variation for several immune traits, not only measuring baseline immunity, but also functional immunological assays (Aguirre-Gamboa et al., 2016; Li et al., 2016). GWAS for defined immunological traits offer exiting and promising opportunities for unravelling genetic variation and fundamental mechanisms in the immune system in human and animal species.

In summary, in this GWAS for KLH-binding NAb and total concentrations of antibodies, we identified three genomic regions: Two regions were suggested on chromosome 9 and chromosome 18 for KLH-binding IgA NAb, and one region was significantly associated on chromosome 4 with KLH-binding IgT NAb, total IgM concentration, and especially with KLH-binding IgM NAb. Further investigation predicted the causal variant on chromosome 4 to be located in *TLR1A*. As far as we know, this is the first time a TLR has been connected to IgM antibody levels in healthy, non-immunized individuals. This suggests a fundamental role of TLR in IgM production, and possibly in B cell development and proliferation. Future

research should confirm the role of *TLR1A*, its role in IgM antibody levels, and its role in general disease resistance in chickens.

Conflict of Interest

This study was in kind funded by Hendrix Genetics. In addition, A.L.J. Vereijken is employed by Hendrix Genetics Research. A.L.J. Vereijken was involved in genotyping and sequencing of the chickens, and imputation of genomic data to the described genomic data sets. These genomic data sets are also of interest for Hendrix Genetics' commercial targets, but this interest did not influence the results in this manuscript in any matter. Except for the delivered data, and the results reported in this manuscript or for other projects, no other shared interests (e.g. employment, consultancy, patents, products) exist between Hendrix Genetics and Wageningen University & Research.

Author Contributions

TB performed the research, analyzed and interpreted the data, and wrote the manuscript. MV analyzed and interpreted the data, and revised the manuscript. JA analyzed and interpreted the data, and revised the manuscript. HP, JP, HB designed the research, interpreted the data, and critically revised the manuscript. AV performed the research, and analyzed and interpreted the data.

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References

See List of references at the end of this PhD thesis.

Supplementary Tables

Supplementary Table 1. Distribution of SNP used in this study over the chicken genome. The table shows: name of the chromosome (chromosomes 1-28 and 30-33, linkage group LGE64, sex chromosomes W and Z, and unplaced), total length of the chromosome (in basepair), the number of SNP on the chromosome, the number of monomorphic SNP (fixed) removed after quality control (step 1), the number of SNP removed after quality control (step 2-4), the number of SNP used in the genome-wide association studies. Table is based on Gallus_gallus-5.0.

		# SNP			
Name	**Total length (bp)**	**All**	**Fixed[a]**	**Cleaned**	**Used**
1	210,673,733	8,651	5,470	757	2,424
2	158,710,615	6,574	4,418	433	1,723
3	116,920,747	4,953	3,261	523	1,169
4	96,543,418	4,036	2,336	358	1,342
5	63,034,728	2,640	1,660	216	764
6	37,500,173	2,089	1,115	179	795
7	38,707,053	2,177	1,332	187	658
8	32,086,491	1,700	898	149	653
9	25,237,187	1,426	770	106	550
10	21,385,625	1,618	941	187	490
11	21,449,822	1,577	1,028	146	403
12	21,002,089	1,640	1,126	232	282
13	19,897,466	1,437	1,028	101	308
14	16,681,244	1,234	736	105	393
15	13,353,506	1,258	940	131	187
16	1,058,524	35	20	5	10
17	11,686,891	1,055	646	163	246
18	11,426,502	1,087	734	129	224
19	10,716,916	1,011	467	137	407
20	15,720,949	1,869	1,246	118	505
21	7,159,517	936	522	103	311
22	5,435,368	473	319	45	109
23	6,289,313	761	401	119	241
24	6,631,702	886	510	89	287
25	3,644,590	240	169	10	61
26	5,546,572	827	502	72	253
27	6,304,838	613	423	34	156
28	5,243,714	796	522	42	232
30	224,321	0	-	-	-
31	168,864	0	-	-	-
32	253,552	0	-	-	-
33	3,756,441	0	-	-	-
LGE64	1,217,975	39	28	1	10
W	7,082,455	11	11	0	0
Z	88,942,393	3,058	2,966	88	333
unplaced	138,546,488	929	508	40	52
Total	1,230,241,782	57,636	37,053	5,005	15,578

[a] Monomorphic SNP

Supplementary Table 2. Estimated genetic correlations (below the diagonal) and phenotypic correlations (above the diagonal) of total antibody (tIgT) concentration, total IgM (tIgM) concentration, total IgA (tIgA) concentration, and total IgG (tIgG) concentration in a WA leghorn chicken population around 16 weeks of age. tIgT, tIgM, tIgA, and tIgG were log_{10}-tranformed for the analyses. SE are shown in parentheses.

	tIgT[a]	**tIgM**	**tIgA**	**tIgG**
tIgT[a]	-	0.39 (0.02)	0.19 (0.03)	0.99 (0.0003)
tIgM	0.24 (0.22)	-	0.18 (0.03)	0.29 (0.02)
tIgA	0.72 (0.19)	0.17 (0.18)	-	0.13 (0.03)
tIgG	0.99 (0.01)	0.12 (0.26)	0.62 (0.23)	-

[a] tIgT = tIgM + tIgA + tIgG

Supplementary Table 3. All suggestively or significantly associated SNP within genomic regions identified in genome-wide association studies for KLH-binding natural antibody (NAb) titers and total antibody concentrations (µg/ml) in a WA leghorn chicken population around 16 weeks of age. Genomic regions were associated for total KLH-binding NAb (IgT) titer, KLH-binding IgM NAb (IgM) titer, total IgM (tIgM) concentration, and KLH-binding IgA NAb (IgA) titer. The table shows: chromosome of associated genomic region, position of significantly or suggestively associated SNP (in basepair), name of the SNP, the -$\log_{10}$(p-value) of the SNP with the associated trait, and the SNP set used. Only significant (FDR = 0.05) or suggestive (FDR = 0.20; *italic*) -$\log_{10}$(p-value)'s are reported. Positions are based on Gallus_gallus-5.0.

Chromosome	Position (bp)	Name	IgT	IgM	tIgM	IgA	SNP set[a]
4	69,587,709	rs313004783	5.21	13.21	6.55		11k
	69,702,481	rs15614874	4.79	13.84	6.36		imputed
	69,703,708	rs14488032		11.62	5.38		imputed
	69,814,286	rs313437715	4.61	12.79	6.9		11k
	69,951,230	rs16742890		10.16			11k
	70,086,585	rs14696113		8.74			imputed
	70,109,615	rs14694747		5.23	4.57		imputed
	70,220,795	rs16756059		8.39			imputed
	70,250,789	rs80589947		8.33			imputed
	70,353,267	rs14488185		6.59			11k
	70,670,047	rs15615299		6.56			11k
	70,689,865	rs16433183		6.04			imputed
	70,769,109	rs14488433		6.11			11k
	71,057,565	rs15615517		6.24			imputed
	71,097,736	rs16433426		5.47			3k+11k
	71,140,058	rs16433455		6.47			imputed
	71,156,953	rs16433473		5.68			imputed
	71,519,305	rs15615695		5.71			imputed
	71,531,594	rs14488824		5.76			imputed
	71,557,148	rs13774302		5.49			11k
	71,585,796	rs14488914		5.78			imputed
	71,591,683	rs13774314		5.47			11k
	71,626,662	rs14488949		5.78			imputed
	71,684,776	rs14488121		5.63			11k
	71,804,632	rs16433788		5.68			imputed
	71,869,873	rs14489012		5.68			imputed
	71,910,272	rs16433853		5.82			imputed
	71,934,160	rs14489032		5.54			imputed
	71,989,867	rs14489048		5.54			imputed
	72,181,481	rs16433969		6.34			imputed
	72,517,503	rs15616020		6.34			imputed
	72,529,232	rs317821928		6.34			imputed
	72,548,431	rs312660474		6.34			imputed
	73,343,240	rs15616529		5.44			imputed
	73,362,701	rs316906071		5.19			11k
9	12,230,193	rs14672112				4.34	11k
	12,261,658	rs15969591				4.58	imputed
18	9,984,590	rs16347444				4.55	imputed
	10,131,465	rs10731438				4.55	11k
	10,154,530	rs14417110				4.55	imputed

[a] SNP genotyped with a 2,740 (3k) SNP set, a 11,173 (11k) SNP set, or imputed.

CHAPTER 5

Antigen-dependent effects of divergent selective breeding for natural antibodies on specific humoral immune responses in chickens

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Abstract

Selective breeding for natural antibodies (NAb) may increase general disease resistance in poultry. NAb are defined as antigen binding antibodies present in individuals without a known previous exposure to this antigen. Higher NAb levels have been associated with a lower mortality in layer chickens. One of the working mechanisms described for NAb is enhancement of specific antibody (SpAb) responses, but the consequences of different NAb levels on immunization are largely unknown. Layer chickens were divergently selected and bred for keyhole limpet hemocyanin (KLH)-binding NAb titers, resulting in a High line and Low line. In this study, we investigated: 1. the correlated response of NAb (selection) on SpAb levels to different antigens, and 2. the effect of immunization of different antigens and NAb selection on NAb levels. The 50 most extreme females from the High line and the 50 most extreme from the Low line of generation 2 were intramuscularly immunized at 33 weeks of age with 1 mL phosphate buffered saline (PBS) containing one of four treatments: (1) negative control (only PBS), (2) the antigen of the selection criterion; 500 µg KLH, (3) a T helper 1 cell-stimulating antigen; 100 µg avian tuberculin purified protein derivative of *Mycobacterium avium* (PPD), or (4) a T helper 2 cell-stimulating antigen; 250 µg human serum albumin (HuSA). SpAb and NAb IgM, and IgG titers in plasma were determined prior to immunization, and weekly for 5 weeks post immunization by indirect ELISA. In addition, antibody affinity was investigated. The results shows that selection for different NAb levels persists with aging of an individual. No differences in NAb response and SpAb response against KLH and PPD were observed as a consequence of different NAb levels (through selection), but increased and prolonged NAb and SpAb responses against HuSA were observed for the High line compared to the Low line. (Selection for) different natural antibody levels did not impair SpAb dynamics of the immunization, and SpAb affinity against KLH, PPD, and HuSA. NAb levels were not, or for only short-term, affected by immunization. We show here that NAb may enhance SpAb responses, but that this effect is also antigen-dependent. We hypothesize that NAb play a role in general disease resistance through enhancement of the humoral adaptive immune response. This might also have consequences for efficiency of vaccination strategies and vaccination management relying on the humoral adaptive immune system.

Keywords: Chicken, Natural antibody, Specific antibody, General disease resistance, Breeding, Keyhole Limpet Hemocyanin (KLH), avian tuberculin purified protein derivative of *Mycobacterium avium* (PPD), Human serum albumin (HuSA)

Introduction

Vaccine development and vaccination management successfully contributed to control of high-impact infectious diseases over the last decades. However economic losses in poultry production are still estimated to range between 10-20% of the gross production value (FAO, 2014). In combination with restriction on (preventive) medicine use due to new legislation, and increased stocking densities and close contact between chickens due to group housing (Hodges, 2009; Neeteson-Van Nieuwenhoven et al., 2016), poultry with a higher general disease resistance is needed. Selective breeding for increased general disease resistance might provide a feasible strategy to reduce disease sensitivity and consequential economic losses and to increase animal wellbeing.

Many selection experiments for immune parameters have been performed in chicken, either selecting for adaptive immunity for specific diseases (Waters, 1945; Pevzner et al., 1981; Bacon et al., 2000; Pinard-Van der Laan et al., 2004; Loywyck et al., 2005; Beaumont et al., 2009; Guimarães et al., 2011) or model antigens (Siegel et al., 1980; Van der Zijpp et al., 1980; Sundaresan et al., 2005), innate immunity (Juul-Madsen et al., 2007; Li et al., 2008), or group longevity (Cheng et al., 2001). However these strategies have, to the best of our knowledge, not or hardly been implemented in commercial poultry breeding, likely because these strategies did not improve general (non-specific) disease resistance or were accompanied with negative side effects.

NAb are defined as antigen binding antibodies present in individuals without a (known) previous exposure to this antigen (Baumgarth et al., 2005). NAb play an essential role in both the innate and adaptive immunity, and link these two arms of the immune system (Matter et al., 2008; Panda et al., 2015). NAb are suggested to be a first line of defense against all types of pathogens (e.g. viruses, bacteria, parasites) in a wide variety of species (f.e. (Kachamakova et al., 2006; Jayasekera et al., 2007; Owen et al., 2014; Panda et al.; Van Altena et al., 2016)). Working mechanisms described for NAb are, but not necessarily limited to: neutralization of pathogens, activation of the antibody-mediated complement pathway, opsonisation, and enhanced antibody responses (see reviews of Panda et al. (2015) and Ochsenbein et al. (2000) for examples). NAb are mainly of the IgM isotype, but can also be of the IgA and IgG isotypes (Baumgarth et al., 2015; Panda et al., 2015). Due to low antibody affinity and polyspecificity (Ochsenbein et al., 2000), NAb might provide a non-antigen-dependent protection to diseases. Previous studies have shown that a lower mortality of layer chickens was associated to high natural antibody (NAb) levels binding keyhole limpet hemocyanin (KLH) (Star et al., 2007a; Sun et al., 2011; Wondmeneh et al., 2015). In addition, NAb are cheap and easy to measure, were estimated to be heritable (Berghof et al., 2015), and in healthy chickens NAb showed a weak correlation with production traits (Van der Klein et al., 2015). Selective breeding for NAb could therefore be a feasible strategy in breeding for improved general disease resistance in chicken.

A white layer chicken population was divergently selected and bred for high and low KLH-binding NAb titers at 16 weeks of age for two generations. This study aimed to

investigate: 1. the correlated response of NAb (selection) on humoral adaptive immune response, e.g. specific antibody (SpAb) levels. 2. the effect of immunization and NAb selection on NAb levels. In order to do so, the 50 most extreme females from the second generation of each NAb selection line (High line and Low line) were intramuscularly (i.m.) immunized at 33 weeks of age with one of four treatments: (1) negative control (PBS), (2) the antigen of the selection criterion; KLH, (3) a T helper 1 (Th1) cell-stimulating antigen; avian tuberculin purified protein derivative of *Mycobacterium avium* (PPD), or (4) a T helper 2 (Th2) cell-stimulating antigen; human serum albumin (HuSA). SpAb and NAb IgM and IgG titers in plasma were determined weekly for 5 weeks post immunization by indirect ELISA. In addition, antibody affinity, as an indication of immune maturation, was investigated. The results are discussed in light of selection for NAb and consequences on vaccination efficiency, and vaccination management.

Materials and Methods

Ethics statement

The selection and immunization experiments were approved by the "Dierexperimentencommissie" (Animal Experiment Committee) of Wageningen University according to Dutch law (Experiment Codes *Selection*: 2012105 & 2013091; Experiment Code *Immunization*: 2014008).

Chicken populations

Unselected base population

The base population, not selected previously for NAb or related traits, consisted of almost 3,700 commercial purebred White Leghorn chickens (approximately 2,400 females, and 1,300 males) from the "WA" line of "Hendrix Genetics". The layer chicken line was selected mainly for egg production, but also other production traits, e.g. traits related to egg quality. The chickens were kept according to standard management of breeding nucleus farms of Hendrix Genetics. Further details about management can be found in Van der Klein et al. (2015).

Plasma of the studied chicken population was collected at 15 weeks of age (males) or 19 weeks of age (females), and stored at −20°C until use. Selection of the breeding candidates was based on total KLH-binding NAb titers in the plasma sample (for measurement: see later). 25 males and 50 females with the highest titers were selected to breed generation 1 of the High line. 25 males and 50 females with the lowest titers were selected to breed generation 1 of the Low line. Each male was mated to 2 females, and each female was mated to only 1 male. The selected males were approximately 36 weeks of age and the selected females were between 50 and 60 weeks of age when used for artificial insemination. Fertilized eggs were collected for approximately 2 weeks for incubation of generation 1 of the selection lines.

NAb selection lines

Incubation of eggs and housing of chickens for the selection lines (from generation 1 onwards) was at research facility "Carus" from Wageningen University & Research according to standard production practices. However per generation some practices might differ, depending on updated management practices or temporary conditions.

Each generation consisted of approximately 600 chickens per line. Plasma samples were collected at 16 weeks of age, and stored at −20°C until use. These plasma samples were used to measure the selection criterion: total KLH-binding NAb titers at 16 weeks of age (for measurement: see later). Within the High line, 25 males and 50 females with the highest titers were selected to breed the next generation of the High line. Within the Low line, 25 males and 50 females with the lowest titers were selected to breed the next generation of the Low line. Chickens were not exchanged between lines. Only 1 male per family, and 2 females per family were allowed within the selected animals to conserve genetic variation as much as possible. Each male was mated to 2 unrelated females (i.e. not mated with sibling or half-sibling), and each female was mated to only 1 unrelated male (i.e. not mated with sibling or half-sibling). The selected chickens were around 30 weeks of age when used for artificial insemination (twice a week). Fertilized eggs were collected for approximately 2.5 weeks for incubation of the next generation of the selection lines.

At hatch, all chickens were sexed, and received individual identification. The two lines were mixed within sex, and were housed within one stable or per sex in two adjacent stables. Chickens were group housed in floor pens with perches and sawdust bedding. Chickens were housed in 7 pens per sex. From approximately 6 weeks of age they were housed in 14 pens in one stable per sex. From approximately 18 weeks of age, selection candidates were individually housed in a cage housing system to allow individual sperm and egg collection.

The light regime was 23 hours of light per day at hatch, and was gradually reduced to 8 hours of light per day at 15 weeks of age, after which the hours of light per day were gradually increased to 16 hours of light at 22 weeks of age. Temperature was set to approximately 35°C at hatch, and was gradually reduced to 24°C at 4 weeks of age, after which temperature was between 15°C and 25°C. Humidity was between 60% and 80% at hatch until 2 weeks of age, after which humidity was between 40% and 80%. Chickens received a standard rearing diet 1 (mixed diet) until 6 weeks of age, and a standard rearing diet 2 until 15 weeks of age (pelleted diet). From approximately 12 weeks of age, a standard laying diet (pelleted diet) was introduced with access to extra grit. All diets are commercially available. Feed and water were provided ad libitum. Once a day a mixed diet was spread in each pen or cage to promote scratching behavior and reduce feather pecking behavior. In addition, chickens had a straw-filled bucket to reduce feather pecking behavior (pens only).

The chickens received obligatory vaccinations against Marek's disease (day 0 of age; i.m.), infectious bronchitis (day 0, day 14, day 56 and, day 115 of age; eye drop), Newcastle disease (day 7, day 28, day 70, and day 84 of age; eye drop), gumboro (day 25 of age; eye drop),

fowl pox (day 84 of age; i.m.), avian encephalomyelitis (day 84 of age; i.m.), and infectious laryngotracheitis (day 84 of age; eye drop).

Study population

At 32 weeks of age the 50 High line females and the 50 Low line females of generation 2 (that were selected as parents for breeding generation 3) were group housed in 4 pens with laying nests, perches, and wood dust bedding. Other housing conditions were as described above. The females were given 1.5 weeks of acclimatization to the changed environment.

Immunization

At 33 weeks of age (day 0), all females were intramuscularly (i.m.) immunized with one of four treatments (randomly assigned within line): 1) 1 ml phosphate buffered saline (PBS; as negative control) (n = 12 chickens/line), 2) 1 ml PBS containing 500 µg KLH (product number H7017, Sigma-Aldrich, St. Louis, MO, USA) (n = 13 chickens/line), 3) 1 ml PBS containing 100 µg avian tuberculin purified protein derivative of *Mycobacterium avium* (PPD; Avian Tuberculin PPD 2500, containing 25.00 I.U./ml with 0.5% phenol (w/v), Institute for Animal Science and Health, Lelystad, the Netherlands) (n = 13 chickens/line), 4) 1 ml PBS containing 250 µg human serum albumin (HuSA; product number A8763, Sigma-Aldrich) (n = 12 chickens/line). Per thigh, 0.5 ml was injected. At day 0 (prior to immunization), and day 7, day 14, day 21, day 28, and day 35 (post i.m. immunization) plasma samples were collected, and stored at −20°C until use.
NAb and SpAb optical density values (OD) of the study population were measured with ELISA procedure (see later) at 16 weeks of age for total KLH-binding immunoglobulins (IgT), and for the KLH-binding immunoglobulin isotypes IgM, and IgG. NAb and SpAb OD of the study population were measured around 33 weeks of age (at day 0, prior to immunization) for KLH-binding IgT, IgM and IgG, PPD-binding IgT, IgM and IgG, and HuSA-binding IgT, IgM and IgG. KLH-binding IgM and IgG, PPD-binding IgM and IgG, and HuSA-binding IgM and IgG were also measured at each of the six time points during the experimental period. Antibody affinity of KLH-binding IgM and IgG, PPD-binding IgM and IgG, and HuSA-binding IgM and IgG were estimated around 33 weeks of age (day 0, prior to immunization) and day 35 post i.m. immunization.

Natural antibody ELISA

NAb OD were determined in individual plasma samples by an indirect two-step ELISA as described by Berghof et al. (2015), and Van der Klein et al. (2015). Briefly, plasma samples were 1:10 pre-diluted with dilution buffer (PBS [10.26 g/L $Na_2HPO_4 \cdot H_2O$, 2.36 g/L KH_2PO_4, and 4.50 g/L NaCl; pH 7.2] containing 0.5% normal horse serum, and 0.05% Tween® 20). Pre-dilutions were stored at 4°C until use the next day, or were frozen at −20°C until use (with a maximum storage time of three months). Flat-bottomed, 96-well medium binding

plates (Greiner Bio-One, Alphen a/d Rijn, The Netherlands) were coated with 2 µg/mL KLH for KLH-binding antibodies, with 16.67 µg/mL PPD for PPD-binding antibodies, or with 4 µg/mL HuSA in 100 µL coating buffer (5.3 g/L Na_2CO_3, and 4.2 g/L $NaHCO_3$; pH 9.6), and incubated at 4°C overnight. After washing for 6 s with tap water containing Tween® 20, plates were tapped dry. The 1:10 pre-dilution of the samples were further diluted with dilution buffer to 1:40, 1:160, 1:640, and 1:2,560 test dilutions, or to 1:30, 1:90, 1:270, and 1:810 test dilutions for HuSA-binding IgG. Duplicate standard positive plasma samples (a pool of male plasmas, see Van der Klein et al. (2015)) were stepwise 1:1 diluted with dilution buffer. The plates were incubated for 1.5 h at room temperature (20-25°C). After washing, plates were incubated with 1:20,000-diluted rabbit-anti-chicken IgG heavy and light chain (IgT) labeled with horse radish peroxidase (PO) (Cat# A30-107P, RRID:AB_67386), or 1:20,000-diluted goat-anti-chicken IgM labeled with PO (Cat# A30-102P, RRID:AB_66857), or 1:40,000-diluted goat-anti-chicken IgG(Fc) labeled with PO (Cat# A30-104P, RRID:AB_66843) (all polyclonal antibodies from Bethyl Laboratories, Montgomery, TX, USA; see also www.antibodyregistry.org), and incubated for 1.5 h at room temperature (20-25°C). After washing, binding of the antibodies to KLH was visualized by adding 100 µL substrate buffer (containing reverse osmosis purified water, 10% tetramethylbenzidine buffer [15.0 g/L sodium acetate, and 1.43 g/L urea hydrogen peroxide; pH 5.5], and 1% tetramethylbenzidine [8 g/L TMB in DMSO]) at room temperature (20-25°C). After 15 min the reaction was stopped with 50 µL of 1.25 M H_2SO_4. OD were measured with a Multiskan Go (Thermo scientific, Breda, The Netherlands) at 450 nm.

Specific antibody ELISA

IgM and IgG SpAb OD were measured at all six time points during the experimental period. SpAb OD were measured as described for natural antibodies, with the following changes: KLH-binding IgM and IgG and HuSA-binding IgG were tested with 1:400, 1:1,600, 1:6,400, and 1:25,600 test dilutions. HuSA-binding IgM was tested with 1:500, 1:2,500, 1:12,500, and 1:62,500 test dilutions. PPD-binding SpAb OD could be determined within the same ELISA for determining NAb OD.

Titer calculation

Antibody titers were calculated as described by Frankena (1987) (taken from De Koning et al. (2015)). Briefly, the OD of the duplicate standard positive plasma samples were averaged for each plate. Logit values of the OD per plate were calculated using:

$$logit\ OD\ =\ ln\left(\frac{OD}{(OD_{max}\ -\ OD)}\right)$$

where OD is the OD of a well, and OD_{max} is the maximum averaged OD of the duplicate standard positive plasma samples. The last positive well (lpw) of the averaged duplicate standard positive plasma sample was set to the sixth dilution. A linear regression line of the logit OD against the respective log_2-dilution values of the averaged duplicate standard

positive plasma samples was determined, which resulted in a regression coefficient β. Titers of the plasma samples per plate were calculated using:

$$titer = \frac{\text{logit OD}_{\text{lpw}} - \left(\text{logit OD}_{\text{sample}} - \beta \times \log_2\left(\text{dilution}_{\text{sample}}\right)\right)}{\beta}$$

were logit OD_{lpw} is the estimated logit OD at the lpw calculated with the estimated linear regression function using the $\log_2$-dilution value of that well, logit OD_{sample} is the logit OD calculated of the OD closest to 50% of OD_{max} for a plasma sample of an individual (OD_{sample}), β is the regression coefficient of the estimated linear regression function of the averaged duplicate standard positive plasma samples, and $\log_2\left(\text{dilution}_{\text{sample}}\right)$ is the $\log_2$-dilution value at which OD_{sample} occurred, as described by De Koning et al. (2015).

Antibody affinity estimation

Antibody affinity of a plasma sample was estimated on the OD of all dilutions above the background threshold of that sample in the NAb ELISA. Per sample, a minimum of 2 OD above the background threshold were required to calculate antibody affinity. Antibody affinity was only estimated for NAb on day 0 (prior to immunization), and NAb and SpAb on day 35.

Antibody affinity was estimated by fitting a log-log regression model through the OD values for each sample separately:

$$OD' = \beta_0 + \beta_1 * \log_2(dilution)$$

where OD' are the natural logarithm of the OD values of the plasma sample, β_0 is the (estimated) intercept of the log-log regression, β_1 is the (estimated) log-log regression coefficient, and $\log_2$(dilution) is the $\log_2$-value of the dilutions of the plasma sample. The β_1-value represent the antibody affinity of the used plasma sample. A higher β_1-value means a higher decrease in OD for increasing dilutions, and therefore a lower antibody affinity. The difference in β_0-values between samples were assumed to be of minimal influence on the antibody affinity estimation. The antibody affinity was estimated with the "=LOGEST()" function in Microsoft® Excel® 2010 v14.0.7180.5002.

Statistical analyses

NAb titers at 16 weeks of age

The statistical model used for estimating line differences on NAb titers at 16 weeks of age was as follows:

$$y_{ij} = \mu + Line_i + e_{ij} \qquad [1],$$

where y_{ij} is the KLH-binding IgT NAb titer, IgM NAb titer, or IgG NAb titer at 16 weeks of age, $Line_i$ is the fixed effect of line (i = high or low), and e_{ij} is the residual term assumed to be distributed as ~$N(0,\mathbf{I}\sigma_e^2)$. Assumed (co)variance structures of the random models terms and the residual term are $\mathbf{I}\sigma_e^2$, in which **I** is an identity matrix, and σ_e^2 is the residual variance.

A plate effect could not be estimated, because the analyzed samples were measured in the complete generation 2-population. Therefore too few selected chickens per plate (at least 5 chickens per plate) were available to estimate a possible plate effect.

NAb titers at 33 weeks of age

The statistical model used for estimating line differences on NAb titers at 33 weeks of age (day 0, prior to immunization) measured in either the NAb ELISA or the SpAb ELISA was as follows:

$$y_{ijk} = \mu + Line_i + plate_j + e_{ijk} \qquad [2],$$

where y_{ijk} is the KLH-binding, PPD-binding, or HuSA-binding IgT NAb titer, IgM NAb titer, or IgG NAb titer at 33 weeks of age in either the NAb ELISA or the SpAb ELISA, $Line_i$ is the fixed effect of line (i = high or low), $plate_j$ is the random effect of the j^{th} plate on which a sample was analyzed (j = 1-8) assumed to be distributed as ~N(0,$\mathbf{I}\sigma^2_{\text{plate}}$), and e_{ijk} is the residual term assumed to be distributed as ~N(0,$\mathbf{I}\sigma^2_{\text{e}}$). Assumed (co)variance structures of the random models terms and the residual term are $\mathbf{I}\sigma^2_{\text{plate}}$, and $\mathbf{I}\sigma^2_{\text{e}}$, in which **I** are identity matrices, σ^2_{plate} is the plate variance, and σ^2_{e} is the residual variance.
The residuals (e_{ijk}) from the nine traits analyzed in model [2], which are corrected for the previously described fixed and random effects, were used for estimating correlations between different antigen-binding (iso)types within IgT, IgM, or IgG.

SpAb titers during the experimental period

The statistical model used for estimating line, and day differences on SpAb titers based on repeated observations during the experimental period was as follows:

$$y_{ijklm} = \mu + Line_i + Day_j + (L \times D)_{ij} + chicken_k + plate_l + e_{ijklm} \qquad [3],$$

where y_{ijklm} is the antigen-binding IgM SpAb titer, or IgG SpAb titer of chickens immunized with the investigated antigen (KLH, PPD, or HuSA), $Line_i$ is the fixed effect of line (i = high or low), Day_j is the fixed effect of day (post i.m. immunization) at which the blood was collected for antibody measurement (j = 7, 14, 21, 28, or 35), $(L \times D)_{ij}$ is the fixed effect of the interaction between $Line_i$ (L) and Day_j (D), $chicken_k$ is the random effect of the k^{th} chicken (k = 1-26 for KLH-binding and PPD-binding SpAb or k = 1-24 for HuSA-binding SpAb) with repeated observations assumed to be distributed as ~N(0,$\mathbf{T}\sigma^2_{\text{chicken}}$), $plate_l$ is the random effect of the l^{th} plate on which a sample was analyzed (l = 1-40) assumed to be distributed as ~N(0,$\mathbf{I}\sigma^2_{\text{plate}}$), and e_{ijklm} is the residual term assumed to be distributed as ~N(0,$\mathbf{I}\sigma^2_{\text{e}}$). Assumed (co)variance structures of the random models terms and the residual term are $\mathbf{T}\sigma^2_{\text{chicken}}$, $\mathbf{I}\sigma^2_{\text{plate}}$, and $\mathbf{I}\sigma^2_{\text{e}}$, in which **T** is the first-order ante-dependence covariance matrix with the individual chicken as the subject and day as the repeated effect, $\sigma^2_{\text{chicken}}$ is the population variance (i.e. the variance between the chickens), **I** are identity matrices, σ^2_{plate} is the plate variance, and σ^2_{e} is the residual variance. An ante-dependence covariance structure

was chosen as optimal covariance structure for subject based on the finite-sample corrected Akaike Information Criteria (AICC) (Wang et al., 2004) and the possibility to converge the model for all tested traits, after testing models with different covariance structures (Toeplitz, unstructured, and unstructured correlation covariance structures). The highest non-significant fixed effect was removed from the model, and this procedure was repeated until all fixed effects were significant or none remained.

Model [3] was also used to assess the significance of $Line_i$ within each separate Day_j (j = 7, 14, 21, 28, or 35).

To adjust for differences at the start of the experiment in order to get a clearer view on SpAb titer differences in response to the treatment, model [3] (excluding non-significant fixed effects) was extended with $\beta_1 \times Cov_{ijkl}$, where Cov_{ijkl} is the single NAb covariate describing the effect of the tested NAb with regression coefficient β_1. Tested NAb covariates were: KLH-binding IgT, IgM, and IgG NAb titers at 16 weeks of age, and the KLH-binding, PPD-binding, and HuSA-binding IgT, IgM, and IgG NAb titers at 33 weeks of age (day 0, prior to immunization).

NAb titers during the experimental period

The statistical model used for estimating line, day, and treatment differences on NAb titers during the experimental period was as follows:

$$y_{ijklmn} = \mu + Line_i + Day_j + Treatment_k + (L \times D)_{ij} + (L \times T)_{ik} + (D \times T)_{jk} + (L \times D \times T)_{ijk} + chicken_l + plate_m + e_{ijklmn} \quad [4],$$

where y_{ijklmn} is the antigen-binding IgM NAb titer, or IgG NAb titer of chickens not immunized with the investigated antigen (KLH, PPD, or HuSA), $Line_i$ is the fixed effect of line (i = high or low), Day_j is the fixed effect of day (post i.m. immunization) at which the blood was collected for antibody measurement (j = 7, 14, 21, 28, or 35), $Treatment_k$ is the immunization treatment (k = PBS, KLH, PPD, or HuSA), $(L \times D)_{ij}$ is the fixed effect of the interaction between $Line_i$ (L) and Day_j (D), $(L \times T)_{ik}$ is the fixed effect of the interaction between $Line_i$ and $Treatment_k$ (T), $(D \times T)_{ij}$ is the fixed effect of the interaction between Day_j and $Treatment_k$, $(L \times D \times T)_{ijk}$ is the fixed effect of the interaction between $Line_i$, Day_j and $Treatment_k$, $chicken_l$ is the random effect of the l^{th} chicken (l = 1-74 for KLH-binding and PPD-binding NAb or l = 1-76 for HuSA-binding NAb) with repeated observations assumed to be distributed as $\sim N(0,\mathbf{T}\sigma^2_{chicken})$, $plate_m$ is the random effect of the m^{th} plate on which a sample was analyzed (m = 1-48) assumed to be distributed as $\sim N(0,\mathbf{I}\sigma^2_{plate})$, and e_{ijklmn} is the residual term assumed to be distributed as $\sim N(0,\mathbf{I}\sigma^2_e)$. Assumed (co)variance structures of the random models terms and the residual term are $\mathbf{T}\sigma^2_{chicken}$, $\mathbf{I}\sigma^2_{plate}$, and $\mathbf{I}\sigma^2_e$, in which **T** is the first-order ante-dependence covariance matrix with the individual chicken as the subject and day as the repeated effect, $\sigma^2_{chicken}$ is the population variance (i.e. the variance between the chickens), **I** are identity matrices, σ^2_{plate} is the plate variance, and σ^2_e is

the residual variance. The highest non-significant fixed effect was removed from the model, and this procedure was repeated until all fixed effects were significant or none remained. Model [4] was also used to assess the significance of $Line_i$, $Treatment_k$, and $(L \times T)_{ik}$ interaction on the j^{th} day by specifying the specific day in the model. The highest non-significant fixed effect was removed from the model, and this procedure was repeated until all fixed effects were significant or none remained.

Antibody affinity

The statistical model used for estimating line difference on SpAb affinity was equal to model [3] with minor changes: y_{ijklm} is the antibody affinity of antigen-specific IgM SpAb, or IgG SpAb of chickens immunized with the investigated antigen (KLH, PPD, or HuSA), Day_j is the fixed effect of day during the experimental period at which the blood was collected for antibody measurement (j = 0, or 35), and $plate_l$ is the random effect of the l^{th} plate on which a sample was analyzed (l = 1-16) assumed to be distributed as ~$N(0,\mathbf{I}\sigma^2_{plate})$. The statistical model used for estimating line, day, and treatment differences on NAb affinity was equal to model [4] with minor changes: y_{ijklmn} is the antibody affinity of IgM NAb, or IgG NAb of chickens not immunized with the investigated antigen (KLH, PPD, or HuSA), Day_j is the fixed effect of day during the experimental period at which the blood was collected for antibody measurement (j = 0 or 35), and $plate_m$ is the random effect of the m^{th} plate on which a sample was analyzed (m = 1-16) assumed to be distributed as ~$N(0,\mathbf{I}\sigma^2_{plate})$.

Miscellaneous

All statistical analyses were done in SAS v9.3 (SAS software by SAS Institute Inc.). All statistical analyses were performed with the PROC MIXED procedure, except for estimation of Pearson correlations which was performed with the PROC CORR procedure. Significance was declared for p-values ≤ 0.05. The exact number of observations per trait (and statistical model) per sampling moment are shown in Supplementary Information (SI) Table 1. In all analyses the random plate effect also accounts for differences between cages, because plate and cage are confounded effects.

Results

Descriptive statistics and differences of Least Squares (LS) means of NAb titers at 16 and 33 weeks of age for model [1] and model [2] are shown in Table 1. The number of observations are shown in SI Table 1. All KLH-binding, PPD-binding, and HuSA-binding NAb types at 16 and 33 weeks of age (day 0, prior to immunization) were significantly higher in the High line compared to the Low line. Titer differences ranged between 1.3 titer points (PPD-binding IgG at 33 weeks of age) and 4.4 titer points (KLH-binding IgT at 16 weeks of age). Titer differences for KLH-binding NAb at 16 weeks of age were almost halved at 33 weeks of age

Table 1. Descriptive statistics of KLH-binding, PPD-binding, and HuSA-binding natural antibody (NAb) titers of the high NAb selection line (High line) and the low NAb selection line (Low Line) measured for total NAb (IgT), and the isotypes IgM, and IgG at 16 weeks of age and 33 weeks of age (n = 50 per line) and differences of Least Squares means (LSM). Shown is average titer High line (SD), average titer Low line (SD), and difference of LSM (SE) for KLH-binding, PPD-binding, and HuSA-binding IgT, IgM, and IgG at 16 weeks or 33 weeks of age. All differences of LSM within NAb and age class were significantly different ($p \leq 0.001$).

Antibody[1] and age	Type	High line	Low line	Difference
KLH-binding NAb	IgT	8.2 (1.0)	3.8 (0.6)	4.4 (0.2)
at 16 weeks of age	IgM	7.2 (0.9)	5.0 (0.8)	2.2 (0.2)
	IgG	7.6 (1.1)	4.1 (0.8)	3.5 (0.2)
KLH-binding NAb	IgT	7.4 (1.3)	5.0 (1.3)	2.4 (0.3)
at 33 weeks of age	IgM	7.3 (0.9)	5.5 (1.1)	1.8 (0.2)
	IgG	6.3 (1.7)	4.0 (1.2)	2.3 (0.3)
PPD-binding NAb	IgT	7.5 (1.1)	6.0 (1.7)	1.5 (0.3)
at 33 weeks of age	IgM	9.6 (1.1)	7.7 (1.3)	1.9 (0.2)
	IgG	7.6 (1.3)	6.3 (1.7)	1.3 (0.3)
HuSA-binding NAb	IgT	5.4 (1.3)	3.8 (1.0)	1.6 (0.2)
at 33 weeks of age	IgM	5.6 (1.2)	4.1 (1.0)	1.4 (0.2)
	IgG	3.5 (1.5)	2.1 (1.4)	1.5 (0.3)

[1] Antigen abbreviations: KLH: keyhole limpet hemocyanin; PPD: avian tuberculin purified protein derivative of *Mycobacterium avium*; HuSA: human serum albumin.

for IgT and IgG, but KLH-binding IgM NAb titer difference was almost equal at 16 weeks of age (2.2 titer point difference) and 33 weeks of age (1.8 titer point difference).

Correlations between NAb titer residuals (corrected for line and plate effects) at 33 weeks of age are shown in Table 2. Correlation between KLH-binding, PPD-binding, and HuSA-binding IgT and IgG were weak and not significant. However, correlations between KLH-binding, PPD-binding, and HuSA-binding IgM were moderate and highly significant. The correlation between KLH-binding IgM and PPD-binding IgM was 0.49. The correlation between KLH-binding IgM and HuSA-binding IgM was 0.63. The correlation between PPD-binding IgM and HuSA-binding IgM was 0.33. Correlations within the High line or Low line were similar to correlations based on all chickens: for KLH-binding IgM and PPD-binding IgM were 0.42 for the High line and 0.54 for the Low line, for KLH-binding IgM and HuSA-binding IgM were 0.66 for the High line and 0.62 for the Low line, and for PPD-binding IgM and HuSA-binding IgM were 0.32 for the High line and 0.36 for the Low line.

The final models without non-significant effects used for repeated measures analysis of SpAb titers are shown in Table 3. Day 0 was analyzed separately from days 7-35 post i.m. immunization in these analyses, because day 0 (prior to immunization) represents NAb levels before immunization and not SpAb levels. Day 0 is therefore considered to represent a different trait. Day 0 analyses are not shown in Table 3. KLH-binding IgM and IgG SpAb titers were not different between lines (Line-effect). KLH-binding IgM and IgG SpAb titers were different in time (Day-effect). PPD-binding IgM and IgG SpAb were significantly higher in the High line compared to the Low line. PPD-binding IgG, but not PPD-binding

Table 2. Correlations between KLH-binding, PPD-binding, and HuSA-binding natural antibody (NAb) titer residuals (corrected for line and plate effects) based on analyses for total NAb (IgT), and the isotypes IgM, and IgG at 33 weeks of age (n = 100). Correlations are based on a selected group of chickens, and might be biased. A row indicates the correlation between antigen-binding NAb of the mentioned (iso)type. Significances of correlations are represented by: †, indicates $p \leq 0.10$; and *, indicates $p \leq 0.001$.**

Antibody[1]	Type	KLH-binding NAb		PPD-binding NAb	
PPD-binding NAb	IgT	0.17	†	-	
	IgM	0.49	***	-	
	IgG	0.08		-	
HuSA-binding NAb	IgT	0.06		0.12	
	IgM	0.63	***	0.33	***
	IgG	0.11		0.06	

[1] Antigen abbreviations: KLH: keyhole limpet hemocyanin; PPD: avian tuberculin purified protein derivative of *Mycobacterium avium*; HuSA: human serum albumin.

IgM, was different in time. HuSA-binding IgM and IgG SpAb were significantly higher in the High line compared to the Low line. HuSA-binding antibodies were also significantly different in time.

The dynamics of SpAb response, and LS means per line per day as a result of the immunizations are shown in Figure 1. The number of observations and the mean (and SD) per trait per line per sampling moment are shown in SI Table 1 and SI Table 2. No difference between lines in SpAb dynamics was observed (Line × Day-interaction). All SpAb, except PPD-binding IgM, show the typical (primary) immune response: the lowest levels of antibodies at day 0 (prior to immunization), the antibody peaks at 7 days post i.m. immunization (for PPD-binding IgG at 14 days post i.m. immunization), and subsequently a steady decline in antibody levels. All NAb at day 0 (prior to immunization) were significantly higher in the High line compared to the Low line (Table 1).

Covariates were added to the final models (without non-significant fixed effects) to investigate potential explanatory effects of these covariates. Significant covariates are shown in Table 3. No significant covariates were found for KLH-binding IgM and IgG SpAb. PPD-binding IgM NAb at 33 weeks of age (day 0, prior to immunization) was significantly positively associated with PPD-binding IgM and IgG SpAb during the experimental period. In addition, PPD-binding IgT and IgG NAb at 33 weeks (day 0, prior to immunization) of age were significantly positively associated with PPD-binding IgG SpAb during the experimental period. HuSA-binding IgM NAb at 33 weeks (day 0, prior to immunization) of age was significantly positively associated with HuSA-binding IgM SpAb during the experimental period. The interaction between line and KLH-binding IgT NAb at 16 weeks of age (the selection criterion) was significantly associated with HuSA-binding IgG SpAb during the experimental period, where the High line was positively influenced by the IgT NAb and the Low line was negatively influenced by IgT NAb.

The final models without non-significant effects used for repeated measures analysis of NAb titers are shown in Table 4. All IgM and IgG NAb were significantly higher in the High line compared to the Low line. No other effects were observed on KLH-binding NAb.

Table 3. Statistical model and significant covariates with regression coefficient for repeated measures analysis of specific antibody (SpAb) titers. Analyzed were KLH-binding, PPD-binding, and HuSA-binding SpAb titers measured at five days (day 7, 14, 21, 28, and 35 post intramuscular immunization) for IgM and IgG isotypes in chickens (n ≈ 25) immunized with the investigated antigen of the SpAb analyses. Tested covariates were KLH-binding IgT, IgM, and IgG NAb titers at 16 weeks of age, and the KLH-binding, PPD-binding, and HuSA-binding IgT, IgM, and IgG NAb titers at 33 weeks of age (day 0, prior to immunization). Significances of model terms are represented by: NS, indicates not significant; †, indicates p ≤ 0.10 (not included in the final model); *, indicates p ≤ 0.05; **, indicates p ≤ 0.01; and *, indicates p ≤ 0.001.**

Antigen[1]	Isotype	Line effect	Day effect	Line x Day interaction	Significant covariates, regression coefficient β_1
KLH-binding	IgM	NS	***	NS	-
	IgG	NS	***	NS	-
PPD-binding	IgM	***	NS	NS	PPD-binding IgM at 33 weeks, $\beta_1 = 0.84$
	IgG	*	***	NS	PPD-binding IgT at 33 weeks, $\beta_1 = 0.74$
					PPD-binding IgM at 33 weeks, $\beta_1 = 0.46$
					PPD-binding IgG at 33 weeks, $\beta_1 = 0.67$
HuSA-binding	IgM	†	***	NS	HuSA-binding IgM at 33 weeks, $\beta_1 = 0.39$
	IgG	**	***	NS	Line x KLH-binding IgT at 16 weeks[2], $\beta_{1,High} = 0.44$ and $\beta_{1,Low} = -0.98$

[1] Antigen abbreviations: KLH: keyhole limpet hemocyanin; PPD: avian tuberculin purified protein derivative of *Mycobacterium avium*; HuSA: human serum albumin.

[2] Total KLH-binding NAb (IgT) titer is the selection criterion for the two NAb selection lines.

PPD-binding NAb were significantly affected by time. HuSA-binding NAb were also influenced by time and immunization (Day × Treatment-interaction).

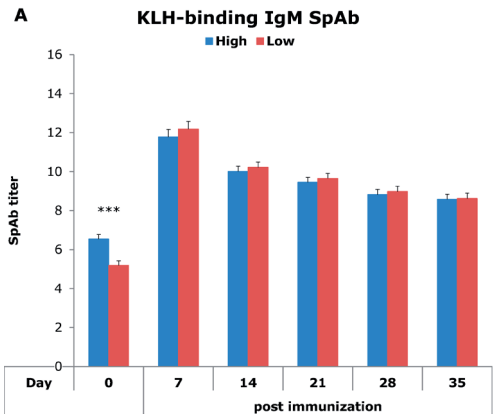
The dynamics of NAb response, and LS means per line per treatment per day as a result of the immunizations are shown in Figure 2. The number of observations and the mean (and SD) per trait per line per sampling moment are shown in SI Table 1 and SI Table 2. All NAb levels were significantly higher in the High line compared to the Low line during the experimental period, although some overlap between lines was observed for HuSA-binding NAb. In addition, KLH-binding NAb and PPD-binding IgG NAb were not influenced by treatment and time. PPD-binding IgM NAb showed a significant influence of immunization (Treatment-effect) at day 7 post i.m. immunization, after which the titers were restored. HuSA-binding NAb showed a significant influence of treatment, which reduced line differences post immunization. Especially the KLH immunized groups showed increased levels of HuSA NAb at day 7 post i.m. immunization, which persisted for HuSA-binding IgG NAb in the Low line during the remainder of the experimental period.

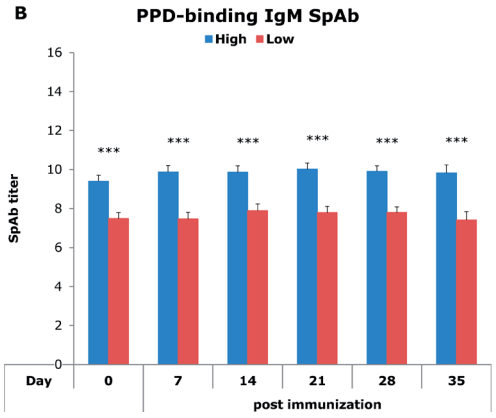
The final models without non-significant effects used for

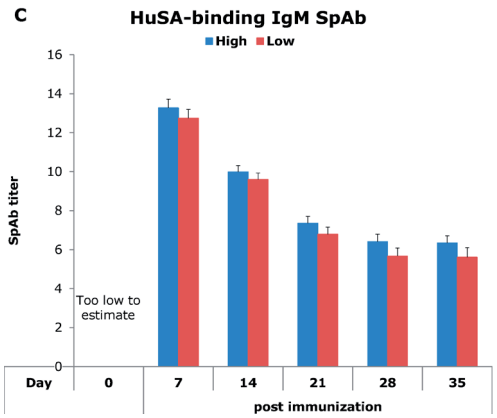
repeated measures analysis of antibody affinity and the LS means for antibody affinity per line per treatment per day are shown in Table 5. The High line showed a higher affinity compared to the Low line for KLH-binding NAb. This was not observed in the group immunized with KLH, likely due to a smaller sample size. No line differences were observed for PPD-binding and HuSA-binding NAb. Increased affinity for IgG SpAb was observed for KLH-binding and HuSA-binding antibodies (compared to NAb), but not for PPD-binding antibodies. Increased affinity for IgM SpAb (at day 35 post i.m. immunization) was only observed for the Low line, but not the High line. This resulted in the loss of significant line difference in IgM antibody affinity. Except for PPD-binding IgG (loss of affinity), no increased affinity was observed for any of the IgM and IgG NAb at day 0 and day 35 post i.m. immunization.

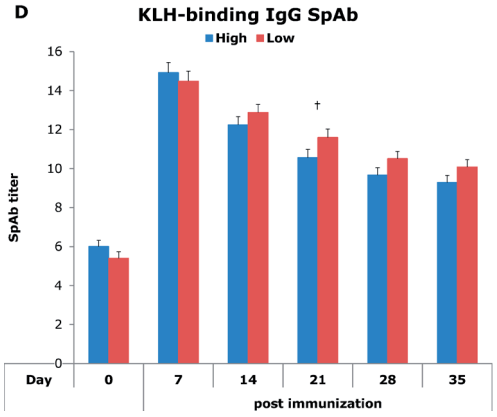
Discussion

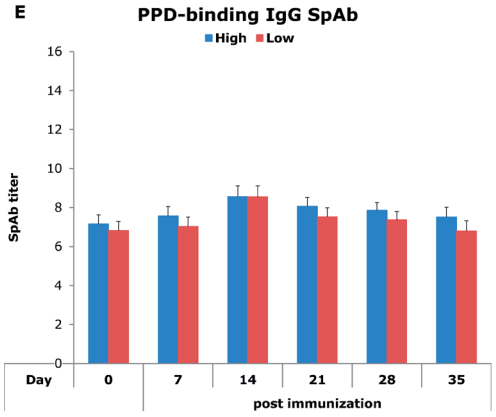
Natural antibodies (NAb) have been associated with increased survival in layer chickens (Star et al., 2007a; Sun et al., 2011; Wondmeneh et al., 2015), suggesting a role of NAb in increased general disease resistance. In addition, NAb were shown to be heritable (Berghof et al., 2015), suggesting that breeding for NAb could provide an alternative strategy to increase general disease resistance in poultry. NAb are also known to have a wide range of (assisting) working mechanisms in the immune system, both at innate levels as well as at adaptive level (see Panda et al. (2015) or Ochsenbein et al. (2000) for an overview). One of these working mechanisms is enhancement of SpAb responses (Boes et al., 1998; Baumgarth et al., 2000; Sinyakov et al., 2002; Stager et al., 2003; Parmentier et al., 2004a; Panda et al., 2015), although decreased SpAb responses through epitope masking and rapid antigen clearance have been reported as well (Na et al., 2006; Parmentier et al., 2008a; Ujvari et al., 2011; Sandmeier et al., 2012). A number of studies investigated the involvement of NAb and immunization on SpAb responses in chickens (Lammers et al., 2004; Parmentier et al., 2008b; Berghof et al., 2010; Cecchini et al., 2016). However, our study investigated the effects of immunization in chickens selected for large differences in NAb levels and their role on SpAb responses. This will provide information on how NAb link different parts of the immunity, and will also provide information on the correlated selection response of SpAb. In addition, this study also investigated the role of immunization on NAb levels, which will provide information of the stability of NAb levels in response to external stimuli. The goal of this study was therefore twofold: 1. investigate the correlated response of NAb (selection) on humoral adaptive immune responses, i.e. specific antibody (SpAb) levels to KLH, PPD, and HuSA, and 2. investigate the effect of immunization and NAb selection on NAb levels. After two generations of selection, the average IgT titer difference between the complete High line and the complete Low line was one titer point (data not shown), meaning that the High line has on average around twice as many KLH-binding IgT NAb at 16 weeks of age compared to the Low line. In order to study the effect of NAb levels on SpAb responses, the 50 most extreme females from both lines were chosen to create a larger difference in average


A
KLH-binding IgM SpAb
High Low
SpAb titer
***
Day 0 7 14 21 28 35
post immunization


B
PPD-binding IgM SpAb
High Low
SpAb titer
*** *** *** *** *** ***
Day 0 7 14 21 28 35
post immunization


C
HuSA-binding IgM SpAb
High Low
SpAb titer
Too low to estimate
Day 0 7 14 21 28 35
post immunization


D
KLH-binding IgG SpAb
High Low
SpAb titer
†
Day 0 7 14 21 28 35
post immunization


E
PPD-binding IgG SpAb
High Low
SpAb titer
Day 0 7 14 21 28 35
post immunization

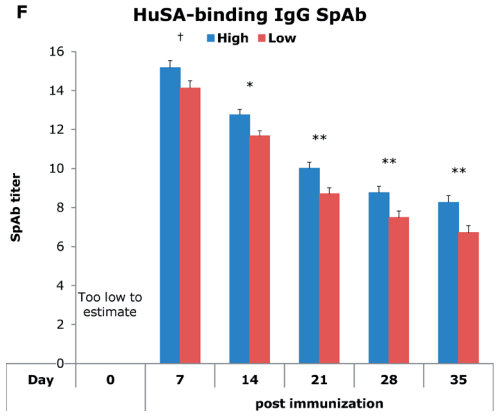

F
HuSA-binding IgG SpAb
† High Low
SpAb titer
Too low to estimate
* ** ** **
Day 0 7 14 21 28 35
post immunization

Figure 1. Dynamics of SpAb response and Least Squares means (and SE) per line per day as a result of immunization. Analyzed were KLH-binding (A, and D), PPD-binding (B, and E), and HuSA-binding (C, and F) natural antibody (NAb) titers (day 0, prior to immunization), and specific antibody (SpAb) titers measured at five days (day 7, 14, 21, 28, and 35 post intramuscular immunization) for IgM (A, B, and C) and IgG (D, E, and F) isotypes in chickens immunized with the investigated antigen (n ≈ 25). HuSA-binding IgM and IgG NAb (day 0) were too low to measure with the SpAb ELISA. Significances of line differences at a specific day are represented by: †, indicates p ≤ 0.10; *, indicates p ≤ 0.05; **, indicates p ≤ 0.01; and *, indicates p ≤ 0.001.**

Antigen abbreviations: KLH: keyhole limpet hemocyanin; PPD: avian tuberculin purified protein derivative of *Mycobacterium avium*; HuSA: human serum albumin.

NAb titers between the lines. IgT and IgG NAb titer differences, but not IgM NAb titer differences, at 16 weeks of age were halved at 33 weeks of age, but still significantly different. In addition to KLH-binding IgT, IgM and IgG NAb, also PPD-binding and HuSA-binding NAb were significantly different at 33 weeks of age. Remarkably, only IgM NAb, but not IgT NAb and IgG NAb, were significantly correlated. Though it should be kept in mind that the correlations are not based on a randomly selected group of chickens, but on a selected group of chickens based on IgT NAb titers at 16 weeks of age. Therefore the estimated correlations might be biased. Nevertheless, these observations suggest that: a) selection for KLH-binding IgT NAb at 16 weeks of age persist over time between selection lines; b) selection for KLH-binding IgT NAb results in line differences for different NAb of different isotypes, suggesting an overall increase of all NAb; and c) selection for line differences is mainly based on (indirect) selection for IgM. With respect to the first objective of this study, different antigens for immunization were chosen. Immunization with KLH was chosen, because of its use in the selection criterion. Immunization with PPD and HuSA were chosen, because of their variety in immune stimulating properties (PPD: Th1 cell-stimulating (Sacco et al., 2002; Forbes et al., 2008); HuSA: Th2 cell-stimulating (f.e. (Ploegaert et al., 2007; Lai et al., 2009; Simon et al., 2016)). No line difference was observed for KLH-binding SpAb, even though the chickens were selected for NAb against this antigen. The chickens did show a significant line difference for PPD-binding SpAb and HuSA-binding SpAb.

KLH is a well-known immunogen (Swerdlow et al., 1996; Harris et al., 1999), used (as adjuvant) in anticancer, and antiviral strategies to boost T cell-dependent responses through antigen-presenting cells (Presicce et al., 2008). Its immunogenic property is particularly contributed to its carbohydrate moiety (Presicce et al., 2008). Also the used dose and KLH's size (monomeric: 345-400 kDA (Söhngen et al., 1997)) compared to PPD (varying sizes, mostly between 6 and 49 kDA (Capsel et al., 2016)) and HuSA (66 kDA (Ghuman et al., 2005)) are likely contributing to the absence of line differences. We hypothesize that the immunogenic properties of KLH overruled possible effects of NAb level differences.

PPD-binding IgG, but not PPD-binding IgM, showed a line difference and an effect of the immunization. However the effect of the immunization was rather small: 1.5-2 titer points. In addition, titers were measured with the NAb ELISA instead of the SpAb ELISA, because of a low SpAb concentration. We hypothesize that PPD's Th1-stimulating nature resulted in a cellular (Th1) response instead of a humoral (Th2)

response. However, effects of different NAb levels on cellular immunity can be expected, but were not part of this study.

In contrast to PPD, HuSA showed a typical humoral (Th2) response with a strong and fast increase in antibodies within a week and a steady decline afterwards. An overall line difference was observed for HuSA-binding IgG SpAb (and a tendency for HuSA-binding IgM SpAb). Though the difference in strength of IgG SpAb at 7 days post immunization tended only to significance, the difference steadily increased afterwards. This indicates no or only a small beneficial effect of NAb levels on the strength of the SpAb response of Th2-antigens, but it also suggests a longer lasting IgG protection and better memory formation due to higher NAb levels.

The dynamics of the immune response between lines did not differ for any of the treatments, suggesting no impairment of the initiation of the immune responses. Previous studies showed that selection for initiation of or strength of the immune response or both has a heritable component (Siegel et al., 1980; Van der Zijpp et al., 1980; Parmentier et al., 2006), which is also linked to NAb levels (Ehrenstein et al., 1998; Parmentier et al., 2004a; Cotter et al., 2005). Differences in the initiation of the immune responses or the strength of the responses might have occurred. More frequent sample collection in especially the first, but also the second week after immunization, could give more insight in the initiation and strength of the immune response in future studies.

Taken together, the data suggests that compared to lower NAb levels, (selection for) higher NAb levels result in a stronger humoral immune response with potentially a better memory formation, but this does not result in difference in dynamics. This is in line with most immunization studies in various species that show a positive association of NAb levels with SpAb levels (Boes et al., 1998; Baumgarth et al., 2000; Parmentier et al., 2004a), or that show a direct positive effect of NAb levels on SpAb levels (Ehrenstein et al., 1998; Lammers et

Table 4. Statistical model for repeated measures analysis of natural antibody (NAb) titers. Analyzed were KLH-binding, PPD-binding, and HuSA-binding NAb titers measured at five days (day 7, 14, 21, 28, and 35 post intramuscular immunization) for IgM and IgG isotypes in chickens (n ≈ 75) without immunization with the investigated antigen of the NAb analyses. Significances of model terms are represented by: NS, indicates not significant; †, indicates p ≤ 0.10 (not included in the final model); *, indicates p ≤ 0.05; **, indicates p ≤ 0.01; and *, indicates p ≤ 0.001. A "." (dot) indicates an effect that cannot be investigated due to a higher order interaction term.**

Antigen[1]	Isotype	Main effect			Interaction			
		Line (L)	Day (D)	Treatment (T)	L x D	L x T	D x T	L x D x T
KLH-	IgM	***	NS	NS	†	NS	NS	NS
binding	IgG	***	NS	NS	NS	NS	NS	NS
PPD-	IgM	***	**	NS	†	NS	NS	NS
binding	IgG	***	†	NS	NS	NS	†	NS
HuSA-	IgM	***	.	.	NS	NS	***	NS
binding	IgG	***	.	.	†	†	**	NS

[1] Antigen abbreviations: KLH: keyhole limpet hemocyanin; PPD: avian tuberculin purified protein derivative of *Mycobacterium avium*; HuSA: human serum albumin.

al., 2004; Cecchini et al., 2016). Some negative associations, possibly through epitope masking or rapid clearance of antigens, were observed as well (Parmentier et al., 2008a; Ujvari et al., 2011; Sandmeier et al., 2012). But, as found in this study, the humoral response and possibly the role of NAb, are strongly dependent on the used antigen-type.

Cecchini et al. (2016) found that IgM NAb, but not IgG NAb, can be used to estimate the potential of the SpAb response. In this study, predictive positive antigen-dependent effects were found for both IgM and IgG NAb, meaning that high NAb levels resulted in higher SpAb levels. However, no clear overall effect of covariates were observed, and a negative relation was even observed for KLH-binding IgT NAb at 16 weeks of age on HuSA-binding IgG SpAb in the Low line. Likely, the selection process is at least partly confounded with the covariates, and the predictive effects of the covariates might therefore be more difficult to detect. Future studies (on unselected populations) should consider testing the predictive effect of these NAb levels on SpAb responses.

With respect to the second objective of this study, immunization effects with different antigens on NAb were not consistent. NAb levels (in mice) have been proposed to be independent of environmental influences (Ochsenbein et al., 2000). KLH-binding NAb showed no effect of immunization or time. PPD-binding IgM NAb, but not IgG NAb, showed a small drop of antibody levels at day 7 post immunization only, especially in the High line. This suggest a short-term depletion of PPD-binding IgM antibodies and a quick recovery. In contrast, HuSA-binding NAb, both IgM and IgG, showed a clear response to immunization. HuSA-binding IgM NAb were elevated only at day 7 post immunization by KLH. Possibly KLH, with its immunogenic characteristics (Swerdlow et al., 1996; Harris et al., 1999), activated all B cells through direct binding to B cells and through T cell-induced secretion of cytokines, which induced a short-term increase in IgM antibody levels. This is known as the bystander response (see f.e. (Baumjohann et al., 2013; Jasiulewicz et al., 2015; Goodridge et al., 2016)). A stronger and longer bystander response of KLH immunization was observed for HuSA-binding IgG NAb. Interestingly, also PPD and PBS induced a bystander response on HuSA-binding IgG NAb, though weaker. This might be the result of some small tissue damage of the i.m. immunization, and consequently the presence of internal damage-associated molecular patterns (DAMP) (Zelenay et al., 2013). DAMP have similar structures and functionality as external pathogen-associated molecular patterns (PAMP) (Zelenay et al., 2013), which are well-known to influence NAb (and SpAb) levels in chickens (Parmentier et al., 2004b; Parmentier et al., 2006; Ploegaert et al., 2007; Star et al., 2007b; Parmentier et al., 2008b; Star et al., 2009; Berghof et al., 2010). But likely the level (or type) of cytokines secreted as a result of the PPD- or PBS-immunization, was lower, and resulted in a weaker response. Overall, we conclude that NAb levels are not effected by immunization, except for HuSA-binding NAb. It remains to be investigated why only HuSA-binding NAb are influenced.

Affinity maturation of antibodies in response to immunization is a well-known phenomenon. In this study, antibody affinity was measured by using ELISA data for estimating antibody levels. This method does not give similar accurate results for antibody affinity as accustomed

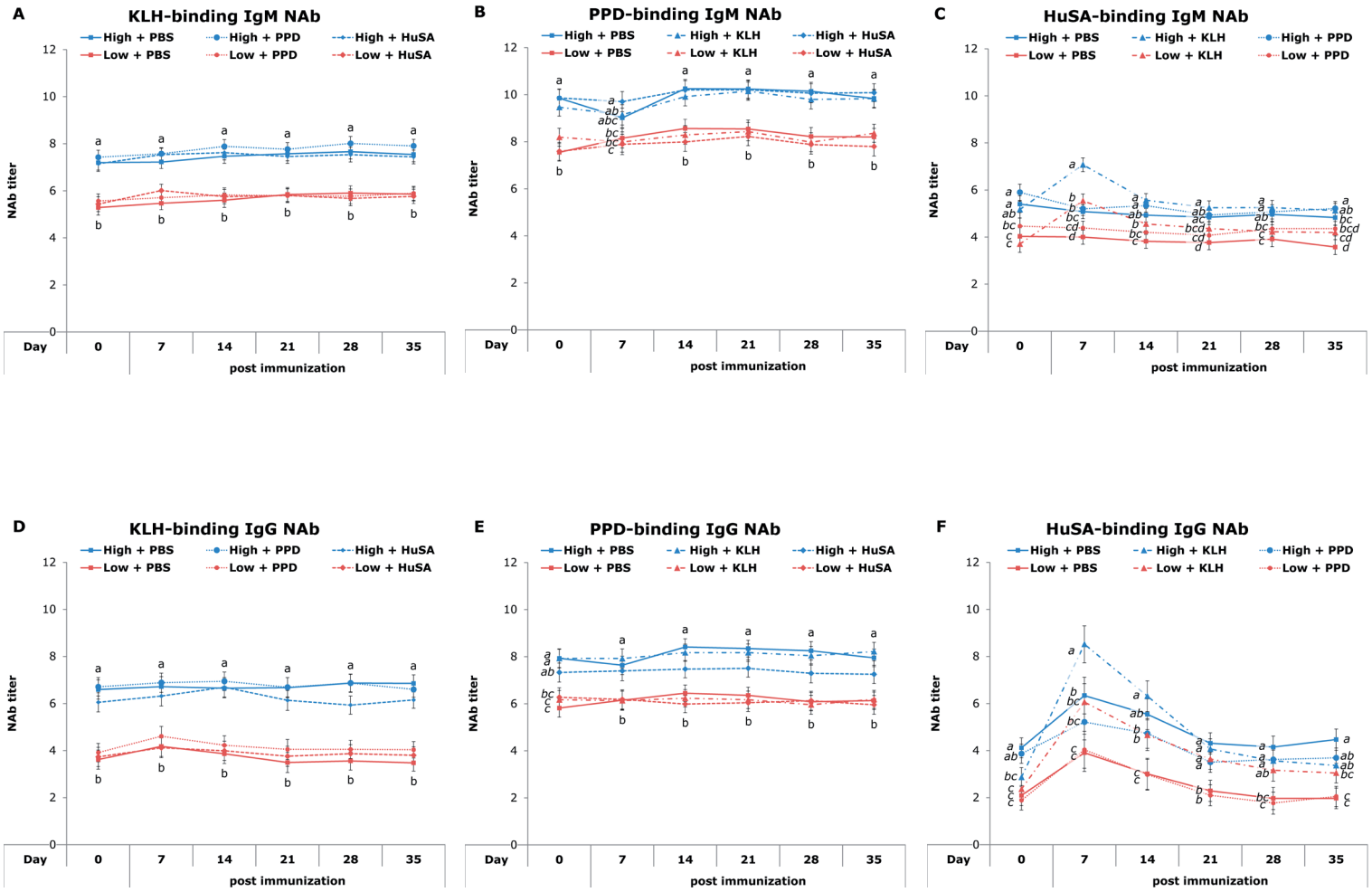

A
KLH-binding IgM NAb
High + PBS
High + PPD
High + HuSA
Low + PBS
Low + PPD
Low + HuSA
NAb titer
Day
0
7
14
21
28
35
post immunization
B
PPD-binding IgM NAb
High + PBS
High + KLH
High + HuSA
Low + PBS
Low + KLH
Low + HuSA
NAb titer
Day
post immunization
C
HuSA-binding IgM NAb
High + PBS
High + KLH
High + PPD
Low + PBS
Low + KLH
Low + PPD
NAb titer
Day
post immunization
D
KLH-binding IgG NAb
High + PBS
High + PPD
High + HuSA
Low + PBS
Low + PPD
Low + HuSA
NAb titer
Day
post immunization
E
PPD-binding IgG NAb
High + PBS
High + KLH
High + HuSA
Low + PBS
Low + KLH
Low + HuSA
NAb titer
Day
post immunization
F
HuSA-binding IgG NAb
High + PBS
High + KLH
High + PPD
Low + PBS
Low + KLH
Low + PPD
NAb titer
Day
post immunization

Figure 2. Dynamics of NAb response and Least Squares means (and SE) per line per treatment per day as a result of immunization. Analyzed were KLH-binding (A, and D), PPD-binding (B, and E), and HuSA-binding (C, and F) natural antibody (NAb) titers measured at six days (day 0, prior to immunization, and day 7, 14, 21, 28, and 35 post intramuscular immunization) for IgM (A, B, and C) and IgG (D, E, and F) isotypes in chickens not immunized with the investigated antigen (n ≈ 75). Significant ($p \leq 0.05$) line differences at a specific day are indicate with different letters (a or b) above or below the means of that specific day. Significant ($p \leq 0.05$) Line × Treatment-differences at a specific day are indicate with different letters (a-d) left or right of the Least Squares means of that specific day.

Antigen abbreviations: KLH: keyhole limpet hemocyanin; PPD: avian tuberculin purified protein derivative of *Mycobacterium avium*; HuSA: human serum albumin.

antibody affinity test for monoclonal antibodies (f.e. Pullen et al. (1986), Schots et al. (1988), Bobrovnik (2003)), but it has the advantage of obtaining additional (indicative) information from a single test on samples with polyclonal antibodies. Indeed, antibody affinity of KLH-binding SpAb, and HuSA-binding SpAb (but not PPD-binding SpAb) were clearly increased compared to NAb, which suggests affinity maturation. This also suggests no impairment of selection on NAb levels on affinity maturation, even though a minor difference in affinity of KLH-binding NAb was observed prior to immunization.

Based on this study, vaccination strategies against infectious diseases might consider NAb levels in individuals (individual or population customized vaccination) for sufficient protection, if the vaccination relies on activation and protection through the humoral adaptive immune system (Th2). Our selection and earlier work (Berghof et al., 2015) indicate a large variety in NAb levels within a population. Low NAb level-individuals might fail to sufficiently build up a protective memory to these types of vaccination. However most vaccinations probably provide an overdose of vaccine. In addition, vaccinations are often directed against viral infectious diseases. A cellular adaptive immunity (Th1) memory formation is in these cases more important. The humoral adaptive immune response of these type of vaccinations does not seem to be impaired based on the tested Th1-dependent antigen, but this study did not examine difference in cellular immunity as a result of (selection for) different NAb levels.

In summary, we conclude that selection for different natural antibody titers at 16 weeks of age remains present at older ages, and possibly has a lifelong effect. However, (selection for) different levels of natural antibodies did not result in differences in natural and specific antibody responses against immunogenic antigens and cellular immunity-skewing (Th1) antigens, but did increase the natural and specific antibody responses against humoral immunity-skewing (Th2) antigens. Therefore natural antibodies might link innate and adaptive immunity, but the influence of natural antibodies on the specific antibody response is antigen-dependent. A role of natural antibodies in general disease resistance in chicken might take place via the Th2 arm of the adaptive immune system. Different natural antibody levels (or selection for different natural antibody levels) did neither impair the specific antibody dynamic of the immunization, nor the specific antibody affinity. Natural antibody levels were not or for short-term affected by immunization. These results imply that vaccination strategies relying on the humoral adaptive immune system might want to consider natural antibody levels

Table 5. Statistical model for and Least Squares (LS) means (and SE) of repeated measures analysis for antibody affinity. Antibody affinity is represented by the β_1-value estimated with a log-level regression model on the optical density (OD) values (above the background threshold) of the tested dilutions of one plasma sample. A higher β_1-value represents a higher decrease in OD for increasing dilutions, and therefore a lower antibody affinity. Analyzed were KLH-binding, PPD-binding, and HuSA-binding antibodies measured at day 0 (prior to immunization), and day 35 (post intramuscular immunization) for IgM and IgG isotypes. IgM NAb/IgM SpAb and IgG NAb/IgG SpAb indicate the analyses of natural antibody (NAb) affinity at day 0 and specific antibody (SpAb) affinity at day 35 for chickens immunized with the investigated antigen of the analyses ($n \approx 25$). IgM NAb and IgG NAb indicate the analyses of NAb affinity at day 0 and NAb affinity at day 35 for chickens not immunized with the investigated antigen of the analyses ($n \approx 75$). A higher antibody affinity is Significances of model terms are represented by: NS, indicates not significant; †, indicates $p \leq 0.10$ (but not included in the final model); *, indicates $p \leq 0.05$; and *, indicates $p \leq 0.001$. A "." (dot) indicates an effect that cannot be investigated due to a higher order interaction term. Significant ($p \leq 0.05$) LS mean differences are indicate with different superscript letters (a-d). Treatment and higher order interaction terms were never significant[2].**

Affinity	Antigen[1]	Line effect	Day effect	Line x Day interaction	β_1 High line (SE)				β_1 Low line (SE)			
					Day 0		Day 35		Day 0		Day 35	
IgM NAb/	PPD	NS	NS	NS	1.33	(0.01)	1.32	(0.01)	1.34	(0.01)	1.33	(0.01)
IgM SpAb	HuSA	NS	†	NS	1.29	(0.02)	1.32	(0.02)	1.29	(0.02)	1.37	(0.02
	KLH	.	.	*	1.29^{b}	(0.02)	1.27^{b}	(0.02)	1.37^{a}	(0.02)	1.28^{b}	(0.02)
IgG NAb/	PPD	NS	†	NS	1.34	(0.02)	1.36	(0.01)	1.31	(0.02)	1.33	(0.01)
IgG SpAb	HuSA	.	.	***	1.29^{a}	(0.02)	1.04^{d}	(0.02)	1.24^{b}	(0.02)	1.10^{c}	(0.02)
	KLH	NS	***	†	1.39^{a}	(0.02)	1.20^{b}	(0.02)	1.43^{a}	(0.02)	1.17^{b}	(0.02)
IgM NAb	PPD	NS	NS	NS	1.33	(0.01)	1.31	(0.01)	1.33	(0.01)	1.31	(0.01)
	HuSA[2]	NS	NS	NS	1.30	(0.02)	1.29	(0.02)	1.31	(0.02)	1.28	(0.02)
	KLH	***	NS	†	1.28^{b}	(0.01)	1.29^{b}	(0.01)	1.35^{a}	(0.01)	1.33^{a}	(0.01)
IgG NAb	PPD	NS	*	NS	1.33^{b}	(0.01)	1.34^{a}	(0.01)	1.32^{b}	(0.01)	1.33^{a}	(0.01)
	HuSA	NS	NS	NS	1.29	(0.03)	1.31	(0.02)	1.25	(0.03)	1.30	(0.02)
	KLH	***	NS	NS	1.37^{b}	(0.02)	1.37^{b}	(0.02)	1.44^{a}	(0.03)	1.46^{a}	(0.02)

[1] Antigen abbreviations: KLH: keyhole limpet hemocyanin; PPD: avian tuberculin purified protein derivative of *Mycobacterium avium*; HuSA: human serum albumin.

[2] Two outliers of the PBS group were removed for the HuSA-binding IgM NAb analysis, resulting in Line × Day × Treatment-interaction to be changed from significant to non-significant.

for sufficient protective immunity. Future work should focus on the effects of NAb (selection) on secondary humoral immune responses and memory formation, as well as on the effect of NAb selection on the cellular Th1 response.

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References

See List of references at the end of this thesis.

Supplementary Information

Supplementary Information Table 1. Number of observations for KLH-binding, PPD-binding, and HuSA-binding natural antibody (NAb) titers of the high NAb selection line (High line) and the low NAb selection line (Low Line) measured for total NAb (IgT), and the isotypes IgM, and IgG per sampling moment. The numbers of observations are shown as 'number of observations High line/number of observations Low line'. Statistical models (as found in Materials & Methods-section) are indicated with numbers in superscript. "-" means not determined. One chicken of the Low line with PPD treatment died after day 0. Two samples could not be collected on day 35: one chicken of the Low line with HuSA treatment, and one chicken of the High line with PBS treatment.

Antibody[a]	Type	16w[a]	33w[a]	Days post immunization				
			0	7	14	21	28	35
KLH-binding SpAb	IgM	-	13/12 [2]	13/13 [3]	13/13 [3]	13/13 [3]	13/13 [3]	13/13 [3]
	IgG	-	13/13 [2]	13/13 [3]	13/13 [3]	13/13 [3]	13/13 [3]	13/13 [3]
PPD-binding SpAb	IgM	-	13/13 [2]	13/12 [3]	13/12 [3]	13/12 [3]	13/12 [3]	13/12 [3]
	IgG	-	13/13 [2]	13/12 [3]	13/12 [3]	13/12 [3]	13/12 [3]	13/12 [3]
HuSA-binding SpAb	IgM	-	2/1[b]	12/12 [3]	12/12 [3]	12/11 [3]	11/9 [3]	10/6 [3]
	IgG	-	1/0[b]	12/12 [3]	12/12 [3]	12/12 [3]	12/12 [3]	12/11 [3]
KLH-binding NAb	IgT	50/50 [1]	50/50 [2]	-	-	-	-	-
	IgM	50/50 [1]	50/50 [2]	37/37 [4]	37/36 [4]	37/36 [4]	37/36 [4]	36/35 [4]
	IgG	50/50 [1]	50/50 [2]	37/37 [4]	37/36 [4]	37/36 [4]	37/36 [4]	36/35 [4]
PPD-binding NAb	IgT	-	50/50 [2]	-	-	-	-	-
	IgM	-	50/50 [2]	37/37 [4]	37/37 [4]	37/37 [4]	37/37 [4]	36/36 [4]
	IgG	-	50/50 [2]	37/37 [4]	37/37 [4]	37/37 [4]	37/37 [4]	36/36 [4]
HuSA-binding NAb	IgT	-	50/50 [2]	-	-	-	-	-
	IgM	-	50/50 [2]	38/38 [4]	38/37 [4]	38/37 [4]	38/37 [4]	37/37 [4]
	IgG	-	50/50 [2]	38/38 [4]	38/37 [4]	38/37 [4]	38/37 [4]	37/37 [4]

[a] Abbreviations: KLH: keyhole limpet hemocyanin; PPD: avian tuberculin purified protein derivative of *Mycobacterium avium*; HuSA: human serum albumin; SpAb: specific antibody; NAb: natural antibody; w: weeks of age.

[b] Observations were not used, because number of observations was too low.

Supplementary Information Table 2. Descriptive statistics for KLH-binding, PPD-binding, and HuSA-binding natural antibody (NAb) and specific antibody (SpAb) titers of the high NAb selection line (High line) and the low NAb selection line (Low Line) measured for the isotypes IgM, and IgG per sampling moment after immunization. The means (SD) are shown as 'mean (SD) of observations High line/mean (SD) of observations Low line'.

Antibody[a]	Type	Treatment	Days post immunization				
			7	14	21	28	35
KLH-binding	IgM	KLH	11.8 (1.7)/12.2 (1.0)	10.0 (0.6)/10.2 (1.2)	9.5 (0.6)/9.7 (1.1)	8.8 (0.6)/9.0 (1.2)	8.6 (0.6)/8.6 (1.2)
SpAb[b]	IgG	KLH	14.9 (2.2)/14.5 (1.5)	12.3 (0.7)/12.9 (2.0)	10.6 (1.0)/11.6 (1.9)	9.7 (0.8)/10.5 (1.7)	9.3 (0.8)/10.1 (1.7)
PPD-binding	IgM	PPD	9.9 (1.1)/7.5 (1.3)	10.0 (0.9)/7.9 (1.2)	10.1 (0.8)/7.8 (1.3)	9.9 (0.9)/7.8 (1.1)	9.8 (1.0)/7.4 (1.8)
SpAb[b]	IgG	PPD	7.8 (1.0)/7.1 (1.8)	8.8 (1.3)/8.6 (2.3)	8.2 (1.0)/7.6 (1.9)	8.0 (1.0)/7.5 (1.5)	7.7 (1.2)/7.0 (1.9)
HuSA-binding	IgM	HuSA	13.3 (1.8)/12.7 (1.3)	10.0 (1.2)/9.6 (1.0)	7.4 (1.5)/6.8 (0.9)	6.4 (1.5)/5.6 (0.6)	6.3 (1.5)/5.6 (0.4)
SpAb[b]	IgG	HuSA	15.2 (1.2)/14.1 (1.4)	12.8 (1.0)/11.7 (0.8)	10.0 (1.0)/8.7 (1.1)	8.7 (1.1)/7.4 (1.1)	8.3 (1.3)/6.7 (1.1)
KLH-binding	IgM	PBS	7.2 (0.8)/5.5 (1.1)	7.5 (0.8)/5.6 (1.2)	7.6 (0.9)/5.8 (1.2)	7.7 (0.7)/5.9 (1.3)	7.7 (0.7)/5.9 (1.3)
NAb		PPD	7.6 (0.7)/5.7 (1.2)	7.9 (1.0)/5.8 (1.1)	7.8 (0.8)/5.8 (1.1)	8.0 (1.1)/5.7 (1.2)	8.0 (1.1)/5.8 (1.2)
		HuSA	7.5 (1.0)/6.0 (0.7)	7.7 (1.1)/5.8 (1.1)	7.5 (0.9)/5.8 (1.2)	7.5 (1.0)/5.6 (1.2)	7.5 (1.0)/5.7 (1.2)
	IgG	PBS	6.7 (1.2)/4.2 (0.8)	6.6 (1.1)/3.8 (0.8)	6.7 (1.1)/3.5 (0.8)	6.9 (1.3)/3.5 (0.9)	6.9 (1.3)/3.6 (0.9)
		PPD	6.9 (1.8)/4.6 (1.3)	7.0 (1.8)/4.2 (1.4)	6.7 (1.8)/4.1 (1.2)	6.9 (1.7)/4.0 (0.9)	6.9 (1.7)/4.1 (0.9)
		HuSA	6.3 (2.2)/4.1 (0.9)	6.6 (1.8)/3.9 (0.9)	6.1 (2.3)/3.8 (0.9)	5.9 (1.9)/3.8 (0.9)	5.9 (1.9)/3.9 (0.9)
PPD-binding	IgM	PBS	9.1 (1.8)/8.0 (2.0)	9.8 (1.6)/8.3 (1.8)	10.1 (1.7)/8.5 (1.9)	9.8 (1.5)/7.9 (2.2)	9.8 (1.5)/8.0 (2.2)
NAb		KLH	9.0 (2.0)/8.2 (1.1)	10.1 (1.0)/8.4 (1.2)	10.2 (1.1)/8.5 (1.2)	10.1 (1.3)/8.2 (1.3)	10.1 (1.3)/8.2 (1.3)
		HuSA	9.7 (0.7)/7.9 (0.9)	10.1 (0.8)/7.9 (0.7)	10.2 (0.9)/8.2 (0.7)	10.1 (0.9)/7.8 (0.9)	10.1 (0.9)/7.9 (0.9)
PPD-binding	IgG	PBS	7.9 (1.4)/6.1 (1.2)	8.2 (1.4)/6.2 (1.2)	8.2 (1.5)/6.2 (1.1)	8.0 (1.7)/5.9 (1.1)	8.0 (1.7)/5.9 (1.1)
NAb		KLH	7.6 (1.7)/6.2 (1.2)	8.4 (0.9)/6.5 (1.2)	8.3 (1.2)/6.4 (1.1)	8.3 (1.1)/6.1 (1.2)	8.3 (1.1)/6.1 (1.2)
		HuSA	7.4 (1.1)/6.2 (1.8)	7.5 (1.1)/6.0 (1.6)	7.5 (1.1)/6.0 (1.6)	7.3 (1.0)/6.1 (1.9)	7.3 (1.0)/6.1 (1.9)
HuSA-binding	IgM	PBS	5.1 (0.8)/4.0 (0.9)	4.9 (0.9)/4.0 (0.9)	4.9 (0.9)/3.9 (0.8)	5.0 (1.1)/3.9 (0.7)	5.0 (1.1)/3.9 (0.7)
NAb		KLH	7.1 (1.3)/5.5 (1.0)	5.6 (1.1)/4.6 (0.9)	5.3 (1.1)/4.4 (1.0)	5.2 (1.5)/4.2 (1.0)	5.2 (1.5)/4.2 (1.0)
		PPD	5.1 (1.0)/4.4 (1.0)	5.4 (1.1)/4.2 (0.9)	5.0 (1.0)/4.1 (1.3)	5.1 (0.8)/4.3 (1.2)	5.1 (0.8)/4.4 (1.2)
	IgG	PBS	6.1 (2.3)/3.1 (1.4)	5.5 (2.2)/2.8 (1.8)	4.3 (2.1)/2.3 (1.0)	4.1 (2.2)/1.9 (0.9)	4.1 (2.2)/1.9 (0.9)
		KLH	7.7 (2.7)/5.2 (2.2)	5.9 (2.7)/4.3 (2.2)	4.0 (1.2)/3.6 (2.3)	3.5 (1.1)/3.1 (2.3)	3.5 (1.1)/3.2 (2.3)
		PPD	4.8 (2.4)/3.6 (2.2)	4.5 (2.0)/2.9 (1.4)	3.5 (1.2)/2.1 (0.7)	3.6 (1.4)/1.7 (0.9)	3.6 (1.4)/1.8 (0.9)

[a] Abbreviations: KLH: keyhole limpet hemocyanin; PPD: avian tuberculin purified protein derivative of Mycobacterium avium; HuSA: human serum albumin; SpAb: specific antibody; NAb: natural antibody; w: weeks of age.

CHAPTER 6

Selective breeding for high natural antibodies reduces mortality after avian pathogenic *Escherichia coli* (APEC) inoculation in chickens

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To be submitted

Abstract

Natural antibodies (NAb) are antibodies recognizing an antigen without previous exposure to that antigen. Keyhole limpet hemocyanin (KLH)-binding natural antibody (NAb) titers in chickens are heritable, and higher levels of KLH-binding NAb have previously been associated with a higher survival during laying period. This suggests that selective breeding for higher NAb levels might increase survival by means of improved general disease resistance. A commercial White Leghorn chicken line was divergently selected and bred for total NAb levels binding KLH at 16 weeks of age for six generations, resulting in a High NAb selection line and a Low NAb selection line. Two infection experiments were performed to test differences in disease resistance: chickens from generation 4 and generation 6 of the NAb selection lines and a Control line (unselected original line) were tested for avian pathogenic *Escherichia coli* (APEC) resistance. Chickens (n = 100 chickens per treatment per line) at 8 days of age received one of four intratracheal inoculations: 1) 0.2 mL phosphate buffered saline (PBS) mock inoculate (experiment 1 and experiment 2), 2) 0.2 mL PBS containing $10^{8.20}$ colony-forming units (CFU)/mL APEC (experiment 1), 3) 0.2 mL PBS containing $10^{6.64}$ CFU/mL APEC (experiment 2), and 4) 0.2 mL PBS containing $10^{7.55}$ CFU/mL APEC (experiment 2). Mortality was recorded during 7 days post inoculation, after which the experiments were ended. Subsequently, morbidity (i.e. colibacillosis lesion scores) was determined on several tissues of the surviving chickens. In experiment 1, a significant lower mortality due to APEC infection was observed for the High line compared to both the Low line, and the Control line. In experiment 2, similar trends were found, but these were mostly not significant, likely due to the lower APEC doses used. Overall, 50-60% reduced mortality was observed in the High line compared to the Low line for all APEC doses. Only the lowest APEC dose resulted in significant different lesion scores: the High line had lower scores compared to the Control line, or Low line, or both. We conclude that selective breeding for high KLH-binding NAb levels at 16 weeks of age increase APEC resistance in early life. This study and previous studies support the hypothesis that KLH-binding NAb might be used as an indicator trait for to selective breed for general disease resistance in an antigen non-specific fashion in layer chickens.

Keywords: Chicken, Natural antibody, Disease resistance, Breeding, *Escherichia coli*, APEC

Introduction

Economic losses in poultry production due to diseases is as high as 10-20% of the gross production value (FAO, 2014). Because of restrictions on (preventive) medicine use in combination with increased close contact between chickens, poultry with a higher general disease resistance are needed (Hodges, 2009; Neeteson-Van Nieuwenhoven et al., 2016). Using a population's natural genetic potential by means of selective breeding for an improved disease resistance might be an important strategy (Star et al., 2007a; Cheng et al., 2013). For decades selection experiments have aimed to improve immunity in chicken, by selecting for adaptive immunity (e.g. Waters, 1945; Siegel et al., 1980; Beaumont et al., 2009), innate immunity (e.g. Juul-Madsen et al., 2007; Li et al., 2008), or group longevity (Cheng et al., 2001). But these strategies have, to the best of our knowledge, not been implemented in commercial poultry breeding, likely because these strategies did not improve general (a-specific) disease resistance or came with negative side effects.

Natural antibodies (NAb) are defined as antigen binding antibodies present in individuals without a (known) previous exposure to this antigen (Baumgarth et al., 2005). NAb play an essential role in both the innate and adaptive immunity, and link these two arms of the immune system (Matter et al., 2008; Panda et al., 2015). NAb can recognize all types of pathogens (i.e. viruses, bacteria, parasites), and assist in the first line of defense against these (e.g. Jayasekera et al., 2007; Owen et al., 2014; Van Altena et al., 2016). NAb are mainly of the IgM isotype, but can also be of the IgA and IgG isotypes (Baumgarth et al., 2015; Panda et al., 2015). NAb might contribute to a broad and general disease resistance, because of their low antibody affinity and polyspecificity (Ochsenbein et al., 2000; Zhou et al., 2007; Wang et al., 2016). Previous studies showed that layer chickens with high natural antibody levels binding keyhole limpet hemocyanin (KLH) were associated with lower mortality (Star et al., 2007a; Sun et al., 2011; Wondmeneh et al., 2015). In addition, NAb are cheap and easy to measure, and are heritable (Berghof et al., 2015). In addition, NAb have weak correlations to production traits in healthy chickens, which suggests that simultaneous selection for production and disease resistance is possible (Van der Klein et al., 2015). Selective breeding for NAb could therefore be a feasible strategy in breeding for increased general disease resistance in chicken.

We divergently selected a white layer chicken population for high and low KLH-binding NAb titers at 16 weeks of age, resulting in a High NAb selection line (High line), and a Low NAb selection line (Low line). Chickens from generation 4 (experiment 1) and generation 6 (experiment 2) of the NAb selection experiment were intratracheally (i.t.) inoculated with different levels of avian pathogenic *Escherichia coli* (APEC) at young age, to investigate protective effects of selective breeding for NAb levels on APEC-induced mortality and morbidity. APEC, a gram negative bacteria, is found in the intestinal microflora of healthy birds (Dho-Moulin et al., 1999). But APEC is an opportunistic pathogen manifesting preferably in the respiratory tract, and causing colibacillosis and eventually death of infected chickens (Dziva et al., 2008; Ewers et al., 2009; Guabiraba et al., 2015). APEC has several

antibiotic resistant mechanisms, and vaccination with heterologous strain are not sufficiently protective (Dho-Moulin et al., 1999; Kariyawasam et al., 2004; Sadeyen et al., 2014). Davies et al. (2009) identified APEC as amenable for genetic improvement of resistance (Davies et al., 2009). APEC is therefore a suitable and relevant candidate disease as a proof-of-principle to test differences in resistance as a result of divergent NAb selection. This paper shows that APEC resistance of the High line was significantly improved compared to the Low line and also to a Control line (not NAb selected, original line). This paper discusses the potential of selection for NAb levels for increased APEC resistance, and general disease resistance.

Materials and Methods

Ethics statement

The selection and infection experiments were approved by the "Dierexperimentencommissie" (Animal Experiment Committee) of Wageningen University & Research according to Dutch law (Experiment Codes *Selection experiment*: 2012105, 2013091, 2014058, & 2014093; Experiment Code *Infection experiment 1*: 2014126; Experiment Code *Infection experiment 2*: 2016057) and the "Central Commissie Dierproeven" (Central Committee Animal Experiments) of the Dutch government (number *Infection experiment 1*: 201521; number *Infection experiment 2*: 2015357).

Chicken populations

Unselected base population and NAb selection lines

A general outline of the selection process is described. More information about the unselected (i.e. not for NAb selected) base population and NAb selection process can be found in Van der Klein et al. (2015) (base population) and Berghof et al. (chapter 5 of this thesis) (NAb selection process).

The unselected base population consisted of almost 3,700 purebred White Leghorn chickens (approximately 2,400 females, and 1,300 males) from the "WA" line of "Hendrix Genetics". Plasma of the studied chicken population was collected at 15 weeks of age (males) or 19 weeks of age (females), and stored at −20°C until use. Selection of the breeding candidates was based on the average total KLH-binding NAb titer in the plasma sample in a 4-step ELISA (for measurement: Berghof et al. (chapter 5 of this thesis)). 25 males and 50 females with the highest titers were selected to breed generation 1 of the High line, and 25 males and 50 females with the lowest titers were selected to breed generation 1 of the Low line. Each male was mated to 2 females, and each female was mated to 1 male.

Incubation of eggs and housing of chickens for the selection lines (from generation 1 onwards) was at research facility "Carus" from Wageningen University & Research according to standard production practices. Each generation consisted of approximately 600 chickens per line. Plasma samples were collected at 16 weeks of age, and stored at −20°C

until use. These plasma samples were used to measure the selection criterion: total KLH-binding NAb titers at 16 weeks of age in a 4-step ELISA and a 8-step ELISA. Selection of parental chickens was done as described for the base population: within a line, the 25 best males and the 50 best females were selected to breed the next generation. Chickens were not exchanged between lines. Each male was mated to 2 unrelated females (i.e. not mated with siblings or half-siblings), and each female was mated to 1 unrelated male (i.e. not mated with siblings or half-siblings).

Study population

Immediately after collecting eggs for generation 4 and generation 6 of the NAb selection lines, a second generation 4 and generation 6 for the infection experiments were produced as described above. In experiment 1, fertilized eggs of the original WA line (not NAb selected) were obtained from Hendrix Genetics breeding facilities as Control line. In experiment 2, 1-day-old chickens of the original WA line were obtained from Hendrix Genetics breeding facilities as Control line in generation 5, and subsequently randomly used to breed the Control line of generation 6 at research facility "Carus".

A climate respiration chamber (CRC) at research facility "Carus", including all required material for the experiment, was disinfected with 1 mL/m^3 Cid 20 (CID LINES N.V.; Ieper, BE) for 1 h. Water pipe lines and drinking nipples were disinfected with MS Oxy-Clean 1.0 (MS Schippers; Bladel, NL) approximately 1:100-diluted with water for 12 h. Eggs were collected, transported to the CRC, disinfected with 5 mL/m^3 Desbest 400 (Frans Veugen Bedrijfshygiëne B.V.; Nederweert, NL) for 1 h, incubated, and hatched. The air influx of the CRC was equipped with a F7 bag filter, a D.carb H11 carbon filter, and a H11 Hepa filter (all filters from Thermo Air; Huizen, NL). The CRC was maintained at overpressure (50-100 Pa) to prevent unfiltered air influx, and to maintain hygiene. More information on CRC can be found in Heetkamp et al. (2015).

For experiment 1, 600 chickens in total (i.e. 200 chickens per line) were selected, preferably 2 males and 2 females per mother. The chickens were randomly distributed over 4 pens in the same CRC of the incubation and hatching.

For experiment 2, 900 chickens in total (i.e. 300 chickens per line) were selected, preferably 2 males and 2 females per mother. The chickens were randomly distributed over 1 pen in the same CRC of the incubation and hatching.

The chickens did not receive any vaccination. Chickens were monitored twice a day from day 0 until day 8. Details on other housing conditions can be found in Berghof et al. (chapter 5 of this thesis).

Sentinel chickens

After selection for the study population, an additional 5 chickens for experiment 1 and an additional 12 chickens for experiment 2 were selected as sentinel chickens. The sentinel chickens were placed in 1 (for experiment 1) or 2 (for experiment 2) separate pen(s) in the

same CRC as the study population, but did not receive any treatment. The chickens did not receive any vaccination. Chickens were monitored twice a day from day 0 until day 8, and twice a day from day 15 until day 22.

Inoculation

At 7 days of age, chickens were randomly assigned to the control (mock) treatment or the APEC treatment(s) in such a way that 100 chickens per treatment per line were assigned. Care was taken to divide treatments within family and within sex, if sufficient chickens per family and per sex were available.
At 8 days of age, all chickens were intratracheally (i.t.) inoculated with their assigned treatment using a 1.0 ml syringe fitted with a blunted anal cannula. For experiment 1, treatments were inoculated by one person (M. G. R. Matthijs), starting with the control treatment and subsequently the APEC treatment per pen. For experiment 1, the end time of the last inoculated chick was taken as starting time for the experiment. For experiment 2, treatments were inoculated by two persons (APEC treatment by M. G. R. Matthijs (low dose, and subsequently high dose), and control treatment by R. M. Dwars). For experiment 2, the time of inoculation per 25 chickens was taken as starting time for these chickens. The control treatment consisted of 0.2 ml phosphate buffered saline (PBS), and the APEC treatment consisted of 0.2 ml PBS containing *Escherichia coli* serotype O78:K80 (strain 506) (a.k.a. APEC). The APEC culture was prepared by making a 1:5 dilution in PBS (experiment 1 and experiment 2) and a 1:50 dilution in PBS (experiment 2) of a 20 h APEC culture, as described by Matthijs et al. (2003). The APEC treatment was prepared and colony forming units (CFU)/mL were determined by "de Gezondheidsdienst voor Dieren" (GD Animal Health; Deventer, the Netherlands) for experiment 1, and by "Veterinair Microbiologisch Diagnostisch Laboratorium" (veterinarian microbiological diagnostic laboratory; Utrecht University; Utrecht, the Netherlands) for experiment 2. Used concentrations of APEC for experiment 1 was $10^{8.20}$ (= 1.60×10^{8}) CFU/mL for the 1:5 dilution, and for experiment 2 were $10^{6.64}$ (= 4.40×10^{6}) CFU/mL for the 1:50 dilution, and $10^{7.55}$ (= 3.55×10^{8}) CFU/mL for the 1:5 dilution.

Data collection

Mortality and Humane endpoints

After inoculation, the chickens were checked every 2 h for the first 4 days post inoculation, and subsequently every 8 h until the end of the experiment (7 days post inoculation). A check consisted of collection of dead chickens, notation of moment of death, and subsequent freezing of these chickens, and observations for chickens complying with the predefined humane endpoint. The humane endpoint was defined based on Marchewka et al. (2013) with minor additions: chickens were euthanized if chickens complied with at least 2 out of 3 requirements for more than 24 h continuously. The requirements were: 1. the chicken is not

able to eat or drink independently, 2. the chicken shows symptoms of paralysis, and 3. the chicken has breathing problems and closed eyes. In addition to the pre-defined requirements, chickens were checked for alertness. Chickens taken out of the experiment were assumed to have died within the time block of the next check, and the time of death was adjusted to the time of the next check. In experiment 1, 5 chickens were taken out (4 Control line chickens and 1 Low line chicken), and in experiment 2, no chickens were taken out.
Mortality was scored as the percentage of chickens alive at a certain moment in hours post inoculation (HPI) divided by the total number of chickens at the start of the experiment. At 15 days of age (7 days post inoculation), all surviving chickens were euthanized per pen.
Necropsy was performed on the chickens that died after the inoculation to exclude causes of death other than APEC. In addition, the findings at post mortem in the chickens that died during the experiment was scored as (suspected) sepsis (e.g. signs of abnormal bleeding, hemorrhage, organ damage), deposition of fibrinopurulent exudate in serosal surfaces of the lumina and air sacs (fibrin deposition), or both. The sex of all birds was verified by checking the reproduction organs.

Morbidity

Morbidity was macroscopically assessed on the surviving chickens after ending the experiment as described by Van Eck et al. (1991). Chickens were randomly and blind scored for colibacillosis lesions by one examiner (M. G. R. Matthijs). Lesion scoring was performed on: the left thoracic air sac, the right thoracic air sac, the pericardium, and the serosal surface of the liver, always in the same order. Severity of the lesion scores were defined as follows: 0: no lesions, 0.5: one pinhead-sized inflammatory spot, 1: two or more pinhead-sized spots, 2: fibrinous patches on various locations, and 3: extensive fibrinous patches (Van Eck et al., 1991). The total lesion score is the sum of scores for the four individual locations. The lesion prevalence was characterized as not infected, airsacculitis, or systemic (Ask et al., 2006b): Chickens with no lesions were considered to be not infected. Chickens with lesions only on the left thoracic air sac, the right thoracic air sac, or both were considered as only airsacculitis (non-systemically infected). Chickens with lesions on the pericardium (pericarditis), the serosal surface of the liver (perihepatitis), or both were considered as systemically infected.
The sex of all birds was verified by checking the reproduction organs.

Sentinel chickens

Blood plasma samples of sentinel chickens were collected at day 22 of age after cervical dislocation. Plasma samples were send to “de Gezondheidsdienst voor Dieren” (GD Animal Health; Deventer, the Netherlands) for presence and quantification of infectious bronchitis-binding antibodies specific for D1466, D274, D8880, M41, and 739B-4/91 and Newcastle disease-binding antibodies by hemagglutination inhibition, *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS) by serum plate agglutination, and MG-binding antibodies and MS-binding antibodies by ELISA.

Necropsy was performed on the sentinel chickens to exclude abnormalities and other diseases besides APEC.

Statistical analyses

Survival analysis

The statistical model used for performing a survival analysis to estimate differences in line, sex, and treatment on mortality was a Cox proportional hazards model (PROC PHREG) (Cox, 1972; Kleinbaum et al., 2012). No PBS inoculated chickens died for both experiments (i.e. no variation to test), and these chickens were left out of the survival analyses. Experiment 1 and experiment 2 were analyzed separately using the following model:

$$h_i(t) = h_0(t) \times \exp[\boldsymbol{X}_i \times \boldsymbol{\beta}] \qquad [1],$$

where $h_i(t)$ is a hazard function for experiment 1 describing the probability at time t (t = 0-170 HPI) for death to occur on the i^{th} APEC inoculated chicken (i = 1-293) or for experiment 2 describing the probability at time t (t = 0-165.4 HPI) for death to occur on the i^{th} APEC inoculated chicken (i = 1-591), $h_0(t)$ is an unspecified baseline hazard function at time t, $\mathbf{X}_i$ is a design matrix containing systematic environmental effects (i.e. predictors (Kleinbaum et al., 2012)) with $\boldsymbol{\beta}$ being the vector with parameters. Investigated predictors for experiment 1 and experiment 2 were $Line_j$ (j = High, Control line, or Low line), Sex_k (k = female, or male), and $Line_j \times Sex_k$. In addition for experiment 1, Pen_m (m = 1-4) was included in the model.

Exact conditional probabilities was calculated for chickens that died at the same moment (ties), according to Kalbfleisch et al. (2011). Chickens that were euthanized at the end of the experiment were censored. The highest non-significant predictor variable was removed from the model, and the model was rerun. This procedure was repeated until all predictor variables were significant or none remained. Assumed proportionality of a hazard function in time was tested by estimating the correlation between the Schoenfeld residuals for a predictor variable and the ranking of individual HPI for chickens that died (Kleinbaum et al., 2012). Stratification of significant predictor variables were investigated by visually checking the survival curves for a similar pattern (no crossing of survival curves) for each class of the investigated predictor variable (Kleinbaum et al., 2012).

Differences in mortality were expressed as hazard ratios (HR) representing the hazard function of a line compared to the hazard function of the reference line, and calculated as the exponent of the respective line regression coefficients, as follows:

$$HR = \frac{h_i(t)^*}{h_i(t)}$$

where HR is the hazard function of a certain line (High line, Control line, or Low line), $h_i(t)^*$ is the hazard function for a chicken of a certain line (High line, Control line, or Low line), and $h_i(t)$ is the hazard function for a chicken of the reference line (here set to High line).

Morbidity

Kruskal-Wallis (KW) tests (PROC NPAR1WAY) were used for estimating difference in line, sex, and treatment on the lesion scores on the left thoracic air sac, on the right thoracic air sac, on the pericardium, on the serosal surface of the liver, on the total lesion score, and on the lesion prevalence. KW tests allow only one class effect to be tested each time, therefore we chose the following strategy to test different class: 1. significance of treatment effects was tested (for experiment 1: PBS inoculation, and $10^{8.20}$ CFU/mL APEC inoculation; for experiment 2: PBS inoculation, $10^{6.64}$ CFU/mL APEC inoculation, and $10^{7.55}$ CFU/mL APEC inoculation). 2. significance of line effects was tested (High line, Control line, and Low line). And 3. significance of sex effects was tested (female, and male). Testing of significances was done on the complete population of experiment 1 or experiment 2 for step 1. Testing of significances was done on the complete population of experiment 1 or experiment 2 for step 2, and 3, unless step 1 or step 2 or both steps was (were) found to be significant. In that case, the population was split up according to that effect (those effects), and sub-populations were tested separately. Testing for significance of effects with two classes was based on a χ^2 with 1 degree of freedom, and testing for significance of effects with three classes was based on a multiple comparison post hoc test (Siegel et al., 1988).

Miscellaneous

All statistical analyses were done in SAS v9.3. Significance was declared for p-values ≤ 0.05, and tendency to significance was declared for p-values ≤ 0.10.

Results

Chickens were either inoculated with PBS or APEC in two separate experiments. Observed symptoms of APEC inoculated chickens compared to PBS inoculated chickens in both experiments were: mortality, reduced growth, reduced alertness, closed eyes, difficulties laying down resulting in standing straight (also when sleeping), difficulties in movement, (signs of) paralysis of the legs, breathing problems, contractions of the abdomen, diarrhea, and dehydration, regardless of used APEC dose. No PBS inoculated chickens died during the experimental period. Sentinel chickens showed no abnormalities, or (signs of) other diseases. Descriptive statistics of mortality in the experiments is shown in Table 1A and Table 1B. The survival plots showing the survival curves are shown in Figure 1.

For experiment 1, 584 chickens were inoculated. PBS was administered to 99 High line chickens, 96 Control line chickens, and 96 Low line chickens. $10^{8.20}$ CFU/mL APEC was administered to 97 High line chickens, 96 Control line chickens, and 100 Low line chickens. The mean hours post inoculation (HPI) for the $10^{8.20}$ CFU/mL APEC treatment group was 111.6 h for the High line, 103.7 h for the Control line, and 109.1 hours for the Low line.

For experiment 1, the hazard ratio (HR) for the $10^{8.20}$ CFU/mL APEC treatment group was (set to) 1 for the High line, 3.3 for the Control line, and 3.0 for the Low line. The HR of the

Table 1A (Experiment 1). Overview of the number of chickens, the number of chickens that died after inoculation, the mean hours post inoculation (HPI) (and SD) of the chicken died, and the hazard ratio (HR) per treatment (inoculated with PBS or $10^{8.20}$ CFU/mL APEC) per line (High NAb selection line, Control line, and Low NAb selection line). Different superscript ([a] or [b]) indicate significant differences in HR.

	PBS			$10^{8.20}$ CFU/mL APEC		
	High	**Control**	**Low**	**High**	**Control**	**Low**
n	99	96	96	97	96	100
($n_{females}/n_{males}$)	(52/47)	(46/50)	(48/48)	(43/54)	(48/48)	(50/50)
n_{dead}	0	0	0	9	25	24
($n_{dead\ females}/n_{dead\ males}$)	(0/0)	(0/0)	(0/0)	(2/7)	(13/12)	(14/10)
HPI_{dead}	-	-	-	111.6	103.7	109.1
(SD)				(51.8)	(42.2)	(44.3)
HR	-[1]	-[1]	-[1]	1[a 2]	3.3[b]	3.0[b]
[95%-CI]					[1.5;7.0]	[1.4;6.4]

[1] PBS inoculated chickens were not analyzed, because no variation was observed (i.e. no PBS inoculated chicken died).

[2] The Control line was set as reference group.

Table 1B (Experiment 2). Overview of the number of chickens, the number of chickens that died after inoculation, the mean hours post inoculation (HPI) (and SD) of the chicken died, and the hazard ratio (HR) per treatment (inoculated with PBS, $10^{6.64}$ CFU/mL APEC, or $10^{7.55}$ CFU/mL APEC) per line (High NAb selection line, Control line, and Low NAb selection line). Different superscript ([a] or [b]) indicate significant differences in HR.

	PBS			$10^{6.64}$ CFU/mL APEC			$10^{7.55}$ CFU/mL APEC		
	High	**Control**	**Low**	**High**	**Control**	**Low**	**High**	**Control**	**Low**
n	100	97	99	101	97	96	98	99	100
($n_{females}/n_{males}$)	(51/49)	(48/49)	(48/51)	(51/50)	(48/49)	(45/51)	(49/49)	(49/50)	(48/52)
n_{death}	0	0	0	6	2	11	6	16	13
($n_{dead\ females}/n_{dead\ males}$)	(0/0)	(0/0)	(0/0)	(1/5)	(1/1)	(6/5)	(2/4)	(6/10)	(4/9)
HPI_{dead}	-	-	-	100.9	97.7	97.2	94.3	110.0	101.6
(SD)				(45.8)	(61.9)	(34.7)	(50.7)	(41.8)	(48.1)
HR	-[1]	-[1]	-[1]	1[ab 2]	0.3[a]	2.0[b]	1[a 2]	2.7[b]	2.2[ab]
[95%-CI]					[0.1;1.7]	[0.7;5.4]		[1.1;7.0]	[0.8;5.8]

[1] PBS inoculated chickens were not analyzed, because no variation was observed (i.e. no PBS inoculated chicken died).

[2] The Control line was set as reference group.

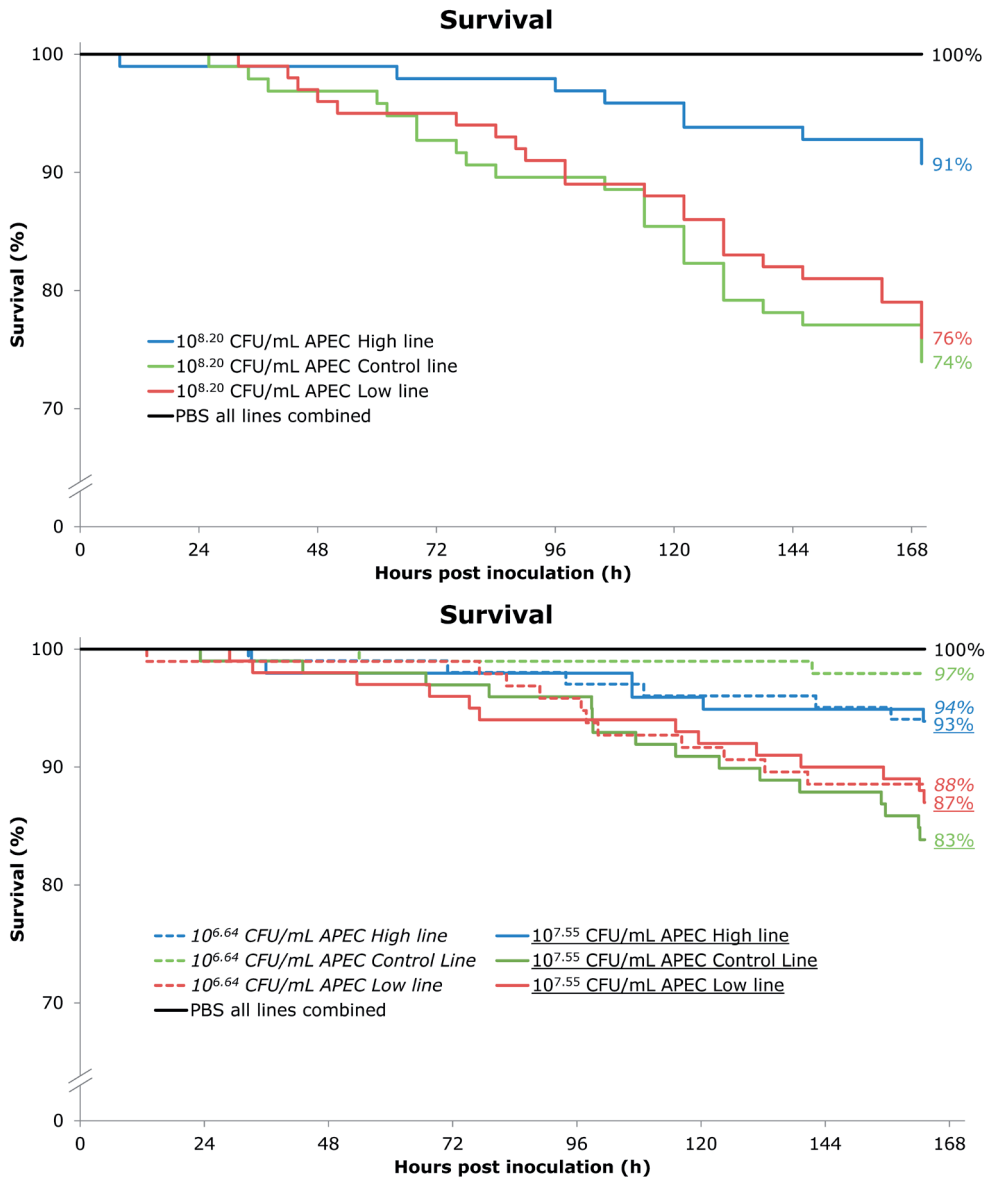


Figure 1 Survival plot showing the hours post inoculation (HPI) and the percentage of chickens alive per High NAb selection line (blue), Control line (green), and Low NAb selection line (red). None of the PBS inoculated chickens died, and therefore the PBS inoculated groups were combined (black). The APEC dose used in experiment 1 (Figure 1A) was $10^{8.20}$ CFU/mL APEC. The APEC doses used in experiment 2 (Figure 1B) were $10^{6.64}$ CFU/mL APEC (dotted line; *italic*), and the $10^{7.55}$ CFU/mL APEC (solid line; underlined).

High line was significantly different from the Control line ($p < 0.01$), and from the Low line ($p = 0.01$). The HR of the Control line was not significantly different with the Low line ($p = 0.71$). The HR were not significantly influenced by sex.

For experiment 2, 887 chickens were inoculated. PBS was administered to 100 High line chickens, 97 Control line chickens, and 99 Low line chickens. $10^{6.64}$ CFU/mL APEC was administered to 101 High line chickens, 97 Control line chickens, and 96 Low line chickens. $10^{7.55}$ CFU/mL APEC was administered to 97 High line chickens, 99 Control line chickens, and 100 Low line chickens. The mean HPI for the $10^{6.64}$ CFU/mL APEC treatment group was 100.9 h for the High line, 97.7 h for the Control line, and 97.2 h for the Low line. The mean HPI for the $10^{7.55}$ CFU/mL APEC treatment group was 94.3 h for the High line, 110.0 h for the Control line, and 101.6 h for the Low line.

For experiment 2, the HR for the $10^{6.64}$ CFU/mL APEC treatment group was (set to) 1 for the High line, 0.3 for the Control line, and 2.0 for the Low line. The HR of the High line was not significantly different with the Control line ($p = 0.19$), and with the Low line ($p = 0.18$). The HR of the Control line was significantly different with the Low line ($p = 0.02$). The HR were not significantly influenced by sex. The HR for the $10^{7.55}$ CFU/mL APEC treatment group was (set to) 1 for the High line, 2.7 for the Control line, and 2.2 for the Low line. The HR of the High line was significantly different from the Control line ($p = 0.04$), but was not significantly different from the Low line ($p = 0.11$). The HR of the Control line was not significantly different with the Low line ($p = 0.54$). The HR tended to be significantly influence by sex ($p = 0.08$) with a HR of 1.9 for males compared to females (set to 1).

The findings at post mortem per chicken that died after inoculation with APEC are shown in Figure 2.

For experiment 1, the findings at post mortem after inoculation with $10^{8.20}$ CFU/mL APEC was devoted to sepsis for 9 chickens, fibrin deposition for 46 chickens, and both for 3 chickens. Sepsis occurred earlier (HPI = 60.7 h) than fibrin deposition (HPI = 118.0 h), while both symptoms occurred in between (HPI = 80.0 h).

For experiment 2, the findings at post mortem after inoculation with $10^{6.64}$ CFU/mL APEC was devoted to sepsis for 6 chickens, fibrin deposition for 7 chickens, and both for 6 chickens. Sepsis occurred earlier (HPI = 67.5 h) than fibrin deposition (HPI = 133.1 h), while both symptoms occurred in between (HPI = 88.9 h). The findings at post mortem after inoculation with $10^{7.55}$ CFU/mL APEC was devoted to sepsis for 14 chickens, fibrin deposition for 7 chickens, and both for 14 chickens. Sepsis occurred earlier (HPI = 69.6 h) than fibrin deposition (HPI = 134.6 h), while both symptoms occurred in between (HPI = 123.6 h).

Descriptive statistics of morbidity (i.e. lesion scores) for the left thoracic air sac, the right thoracic air sac, the pericardium, and the serosal surface of the liver in the experiments are shown in Table 2A and Table 2B. The average lesion scores for the left thoracic air sac, the right thoracic air sac, the pericardium, the serosal surface of the liver in the experiments, the distribution of the total lesion score, and the frequency of lesion prevalence are shown in Figure 3A and Figure 3B.

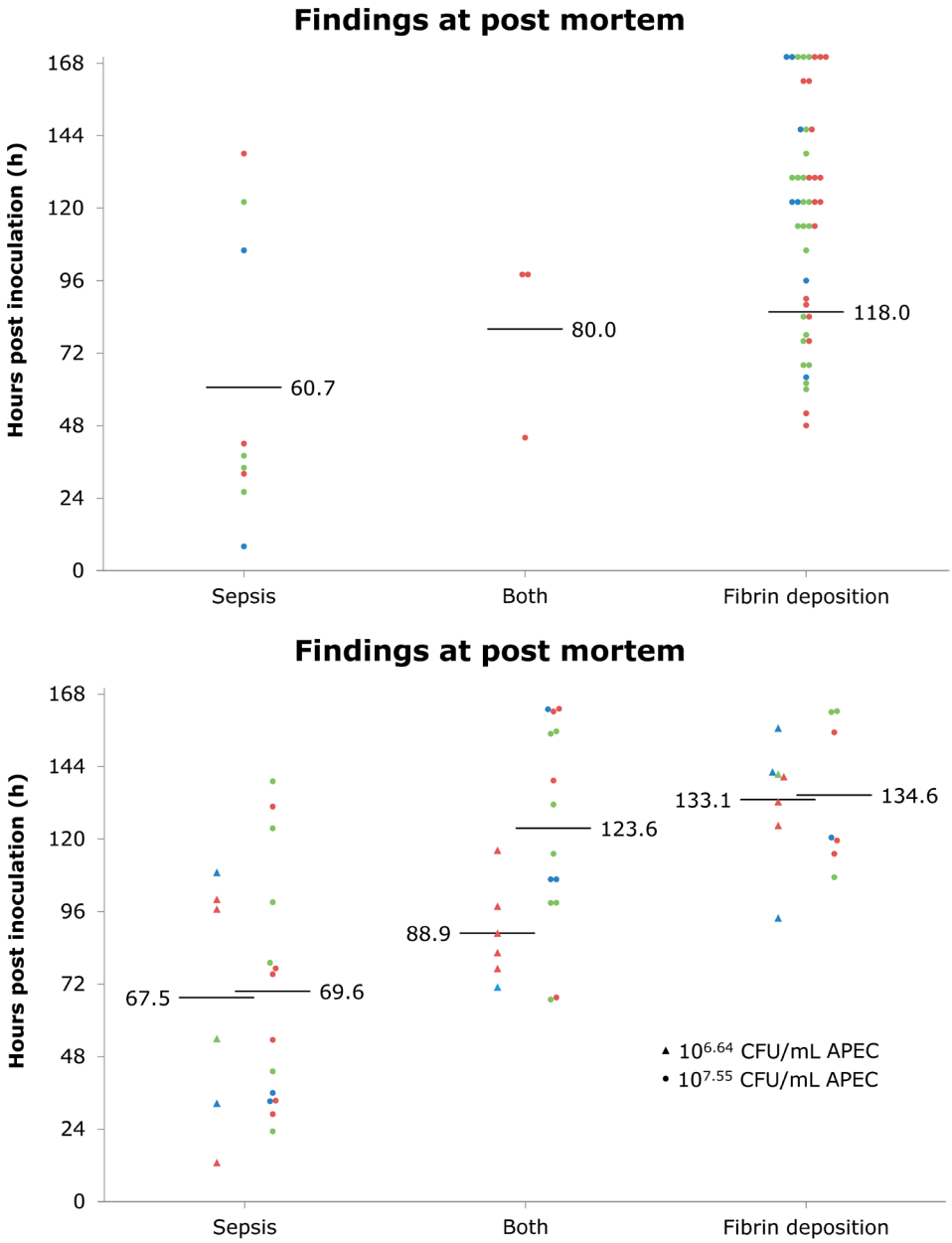


Figure 2. The findings at post mortem of the chickens that died after APEC inoculation based on observations during necroscopy. Shown is the findings at post mortem (sepsis, deposition of fibrinopurulent exudate in serosal surfaces of the lumina, and air sacs (fibrin deposition), or both) and the hours post inoculation (HPI) the chicken died per High NAb selection line (blue), Control line (green), and Low NAb selection line (red). Averages are indicate with a black solid line. The APEC dose used in experiment 1 (Figure 2A) was $10^{8.20}$ CFU/mL APEC. The APEC doses used in experiment 2 (Figure 2B) were $10^{6.64}$ CFU/mL APEC (triangle), and the $10^{7.55}$ CFU/mL APEC (circle).

Table 2A (Experiment 1). Overview of morbidity per treatment (PBS inoculation, or 108.20 CFU/mL APEC inoculation) per line (High NAb selection line, Control line, and Low NAb selection line) by scoring colibacillosis lesions per surviving chicken. Shown is the total number of chickens scored per treatment per line, and the number of chickens with a lesion scorea on the left thoracic air sac (LA), the right thoracic air sac (RA), the pericardium, and the serosal surface of the liver (liver) per treatment per line.

		PBS			$10^{8.20}$ CFU/mL APEC		
		High	Control	Low	High	Control	Low
n_{scored}		99	96	96	88	71	76
LA	0	91	81	86	0	1	1
	0.5	6	11	2	0	0	1
	1	2	4	6	3	0	4
	2	0	0	2	11	12	11
	3	0	0	0	74	58	59
RA	0	93	86	90	0	1	1
	0.5	4	4	6	0	0	0
	1	2	6	0	0	0	2
	2	0	0	0	6	5	9
	3	0	0	0	82	65	64
Pericardium	0	99	95	95	0	1	2
	0.5	0	0	1	0	0	0
	1	0	1	0	0	0	1
	2	0	0	0	5	3	6
	3	0	0	0	83	67	67
Liver	0	99	96	96	1	3	4
	0.5	0	0	0	1	0	0
	1	0	0	0	10	4	7
	2	0	0	0	6	6	11
	3	0	0	0	70	58	54

[a] Severity of the lesion scores were defined as follows: 0: no lesions, 0.5: one pinhead-sized inflammatory spot, 1: two or more pinhead-sized spots, 2: fibrinous patches on various locations, and 3: extensive fibrinous patches (Van Eck et al., 1991).

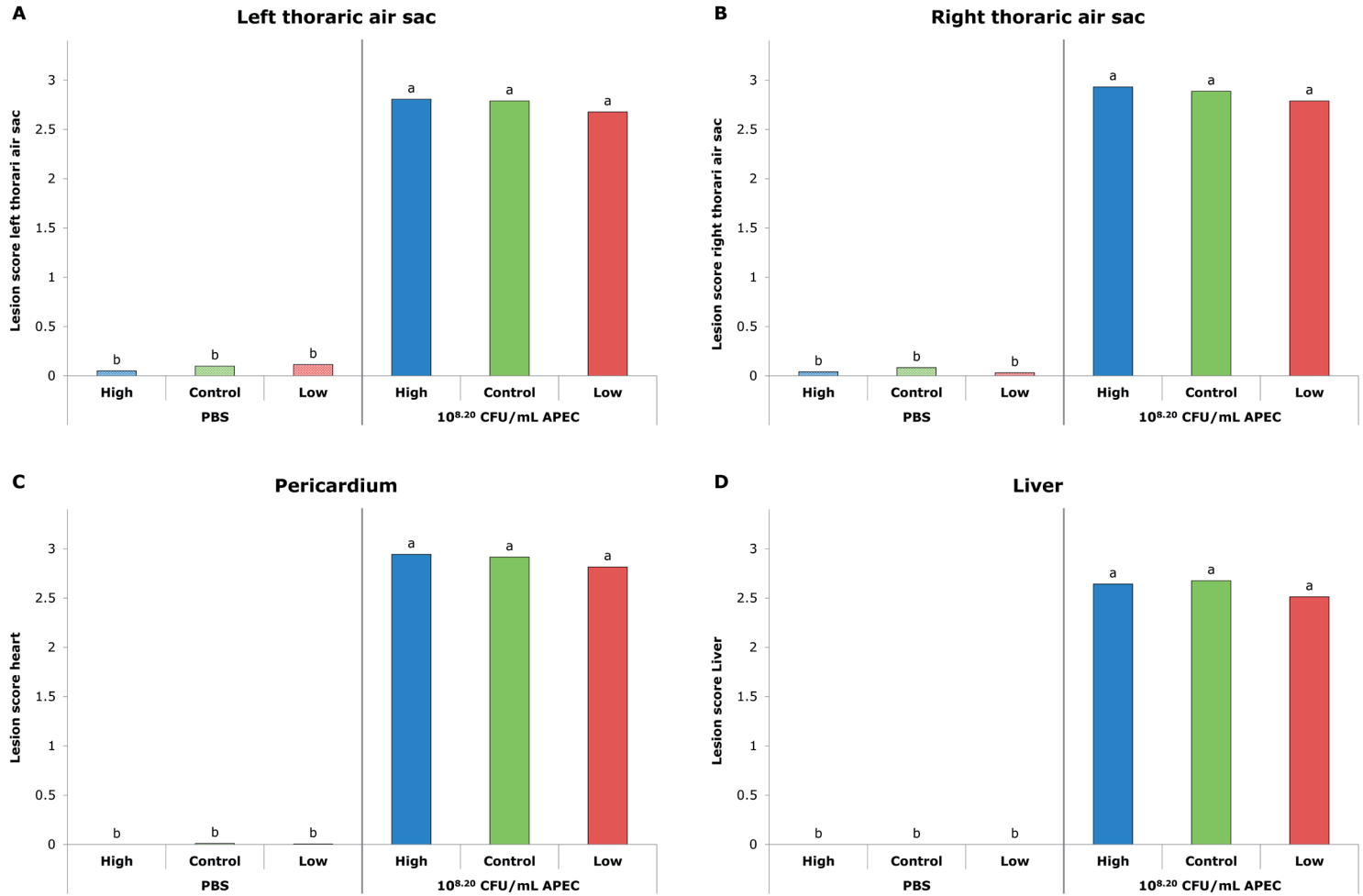
For experiment 1, a significant treatment effect was observed. PBS inoculated chickens were mostly not infected (score = 0): 90% of the High line chickens, 81% of the Control line chickens, and 88% of the Low line chickens. $10^{8.20}$ CFU/mL APEC inoculated chickens were mostly maximally infected (score = 3): 100% of the High line chickens, 99% of the Control line chickens, and 99% of the Low line chickens. Lesions were not significantly different between lines.

For experiment 2, a significant treatment effect was observed in a dose-response relation with the lowest average lesion scores for PBS inoculated chickens (mostly score = 0), in between average lesion scores for the $10^{6.64}$ CFU/mL APEC inoculated group, and the highest average lesion scores for $10^{7.55}$ CFU/mL APEC inoculated chickens. PBS inoculated chickens were mostly not infected (score = 0): 79% of the High line chickens, 79% of the Control line chickens, and 86% of the Low line chickens. APEC inoculated chickens were mostly maximally infected (score = 3): for $10^{6.64}$ CFU/mL, 81% of the High line chickens, 95% of the Control line chickens, and 85% of the Low line chickens, and for $10^{7.75}$ CFU/mL APEC,

Table 2B (Experiment 2). Overview of morbidity per treatment (PBS inoculation, $10^{6.64}$ CFU/mL APEC inoculation, or $10^{7.55}$ CFU/mL APEC inoculation) per line (High NAb selection line, Control line, and Low NAb selection line) by scoring colibacillosis lesions per surviving chicken. Shown is the total number of chickens scored per treatment per line, and the number of chickens with a lesion score[a] on the left thoracic air sac (LA), the right thoracic air sac (RA), the pericardium, and the serosal surface of the liver (liver) per treatment per line.

		PBS			$10^{6.64}$ CFU/mL APEC			$10^{7.55}$ CFU/mL APEC		
		High	Control	Low	High	Control	Low	High	Control	Low
n_{scored}		100	97	99	95	94	85	92	83	87
LA	0	86	86	85	26	12	22	7	2	6
	0.5	14	10	13	13	14	4	6	3	0
	1	0	1	1	21	18	19	5	3	8
	2	0	0	0	19	28	21	27	25	29
	3	0	0	0	16	22	19	47	50	44
RA	0	89	84	95	22	8	13	2	0	3
	0.5	11	11	4	14	5	5	3	1	0
	1	0	2	0	10	21	14	5	2	0
	2	0	0	0	21	24	19	20	13	16
	3	0	0	0	28	36	34	62	67	68
Pericardium	0	99	97	99	60	46	44	20	9	11
	0.5	0	0	0	0	2	0	0	0	1
	1	0	0	0	10	12	10	8	6	9
	2	1	0	0	9	16	10	15	20	15
	3	0	0	0	16	18	21	49	48	51
Liver	0	100	97	98	46	30	25	10	2	6
	0.5	0	0	1	9	2	6	3	1	2
	1	0	0	0	3	6	8	2	2	5
	2	0	0	0	9	21	15	14	13	14
	3	0	0	0	28	35	31	63	65	60

[a] Severity of the lesion scores were defined as follows: 0: no lesions, 0.5: one pinhead-sized inflammatory spot, 1: two or more pinhead-sized spots, 2: fibrinous patches on various locations, and 3: extensive fibrinous patches (Van Eck et al., 1991).


A
Left thoraric air sac
Lesion score left thorari air sac
B
Right thoraric air sac
Lesion score right thorari air sac
C
Pericardium
Lesion score heart
D
Liver
Lesion score Liver
0
0.5
1
1.5
2
2.5
3
a
b
High
Control
Low
PBS
10^8.20 CFU/mL APEC

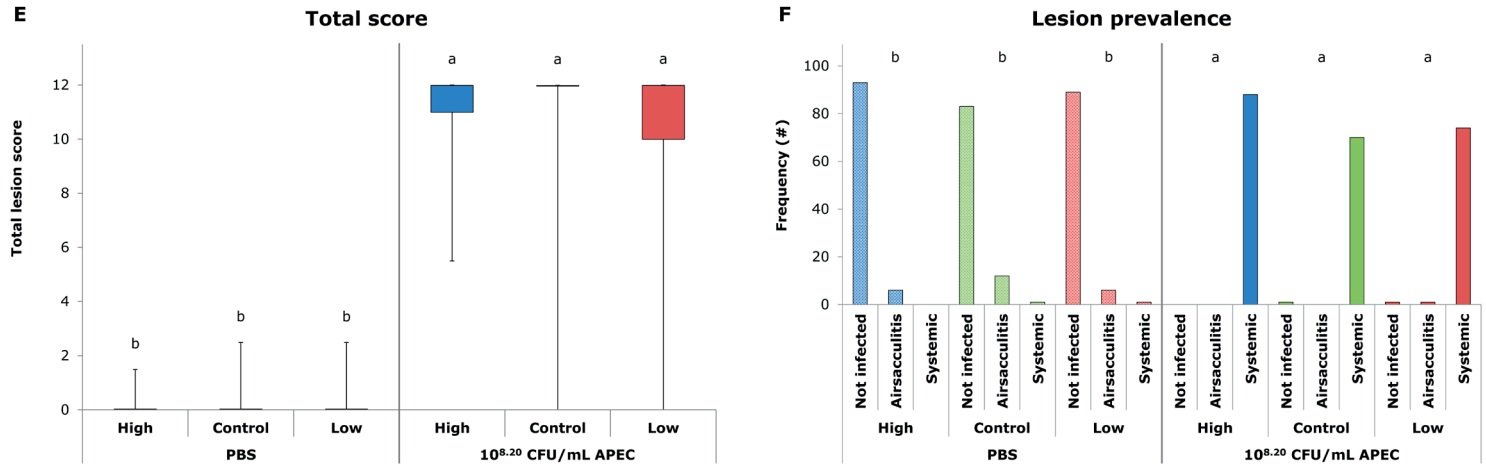
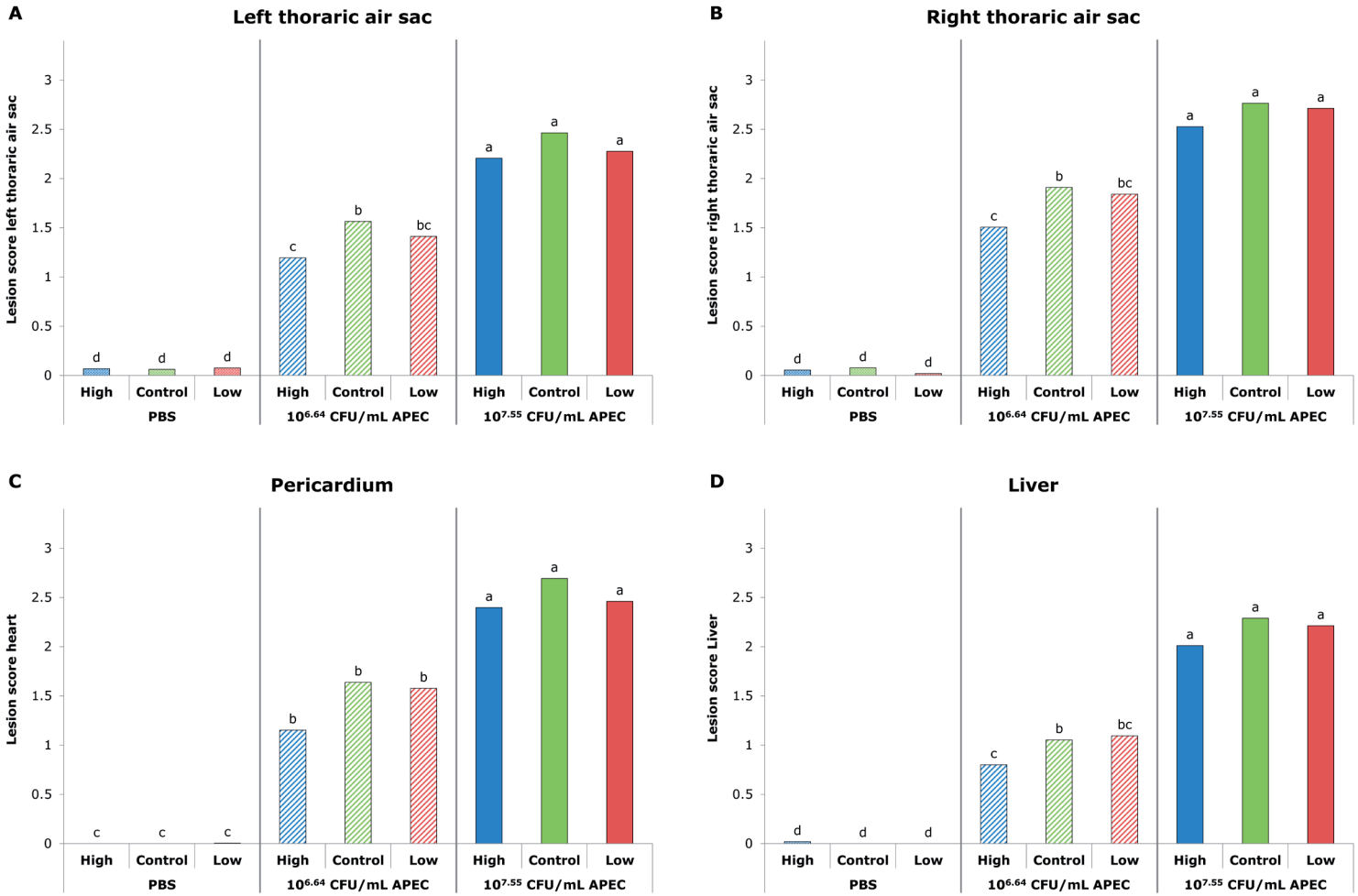


Figure 3A (Experiment 1). Morbidity per treatment (PBS inoculation, or $10^{8.20}$ CFU/mL APEC inoculation) per line (High NAb selection line, Control line, and Low NAb selection line) by scoring colibacillosis lesions per surviving chicken. Severity of the lesion scores were defined as follows: 0: no lesions, 0.5: one pinhead-sized inflammatory spot, 1: two or more pinhead-sized spots, 2: fibrinous patches on various locations, and 3: extensive fibrinous patches (Van Eck et al., 1991). The total lesion score is the sum of scores for the four individual locations. Chickens with no lesions were considered to be not infected. Chickens with lesions only on the left thoracic air sac, the right thoracic air sac, or both were considered as only airsacculitis (non-systemically infected). Chickens with lesions on the pericardium (pericarditis), the serosal surface of the liver (perihepatitis), or both were considered as systemically infected. Shown is: the average lesion per treatment per line on (A) the left thoracic air sac (LA), (B) the right thoracic air sac (RA), (C) the pericardium, (D) the serosal surface of the liver (liver) per treatment per line, (E) the boxplot per treatment per line for total lesion score, and (F) the frequency of lesion prevalence per treatment per line. Different superscript (^{a} or ^{b}) indicate significant differences in lesion scores or frequencies.


A
Left thoraric air sac
Lesion score left thoraric air sac
B
Right thoraric air sac
Lesion score right thoraric air sac
C
Pericardium
Lesion score heart
D
Liver
Lesion score Liver
High
Control
Low
PBS
10^6.64 CFU/mL APEC
10^7.55 CFU/mL APEC

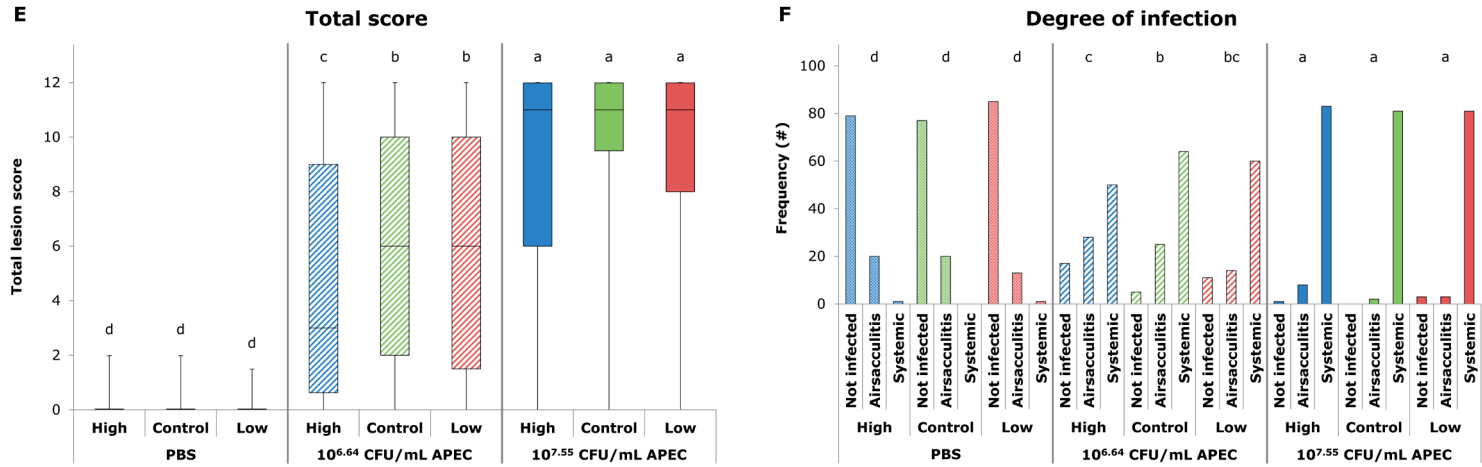


Figure 3B (Experiment 2). Morbidity per treatment (PBS inoculation, $10^{6.64}$ CFU/mL APEC inoculation, or $10^{7.55}$ CFU/mL APEC inoculation) per line (High NAb selection line, Control line, and Low NAb selection line) by scoring colibacillosis lesions per surviving chicken. Severity of the lesion scores were defined as follows: 0: no lesions, 0.5: one pinhead-sized inflammatory spot, 1: two or more pinhead-sized spots, 2: fibrinous patches on various locations, and 3: extensive fibrinous patches (Van Eck et al., 1991). The total lesion score is the sum of scores for the four individual locations. Chickens with no lesions were considered to be not infected. Chickens with lesions only on the left thoracic air sac, the right thoracic air sac, or both were considered as only airsacculitis (non-systemically infected). Chickens with lesions on the pericardium (pericarditis), the serosal surface of the liver (perihepatitis), or both were considered as systemically infected. Shown is: the average lesion per treatment per line on (A) the left thoracic air sac (LA), (B) the right thoracic air sac (RA), (C) the pericardium, (D) the serosal surface of the liver (liver) per treatment per line, (E) the boxplot per treatment per line for total lesion score, and (F) frequency of the lesion prevalence per treatment per line. Different superscript (^{a} - ^{d}) indicate significant differences in lesion scores or frequencies.

98% of the High line chickens, 98% of the Control line chickens, and 92% of the Low line chickens. Lesions were not significantly different between lines for the PBS inoculated chickens, and the $10^{7.55}$ CFU/mL APEC inoculated chickens. However for the $10^{6.64}$ CFU/mL APEC inoculated chickens, lesions (except pericardium) were significantly lower for the High line compared to the Control line, and tended to be significantly lower for the High line compared to the Low line.

Discussion

High KLH-binding NAb levels were previously associated with lower mortality during the laying period of chickens (Star et al., 2007a; Sun et al., 2011; Wondmeneh et al., 2015). NAb are known as a first line defense (Panda et al.; Jayasekera et al., 2007; Owen et al., 2014). In addition, KLH-binding NAb levels are cheap and easy to measure, are heritable (Berghof et al., 2015), and do not seem to influence production traits in healthy individuals (Van der Klein et al., 2015). Selective breeding for NAb could therefore be a feasible strategy in breeding for improved general disease resistance in chicken. We divergently selected and bred two KLH-binding NAb selection lines: a High NAb-selection line and a Low NAb selection line based on NAb titers at 16 weeks of age. We inoculated both selection lines, and a not NAb-selected Control line (representing the original parental line) in two separate experiments with different avian pathogenic *Escherichia coli* (APEC; serotype O78:K80, strain 506) doses to investigate line differences in disease resistance.

Colibacillosis is any local or systemic infection caused entirely or partly by APEC (Barnes et al., 2008). It is one of the main causes of economic loss in the poultry industry worldwide (Barnes et al., 2008). APEC is commonly found in the gastrointestinal tract of healthy birds (Dho-Moulin et al., 1999), but it has opportunistic pathogenic properties. Transmission of APEC occurs, amongst other, via feces, and dust (Charlton, 2006), and it can thereby manifest in the respiratory tract, spread to various organs, and eventually lead to death (Dziva et al., 2008; Ewers et al., 2009; Guabiraba et al., 2015). APEC can be antibiotic resistant, and vaccination with a heterologous strain do not provide sufficient protection (Dho-Moulin et al., 1999; Kariyawasam et al., 2004; Sadeyen et al., 2014). Most economic losses of APEC are associated with broiler production, which is therefore the main study model (Barnes et al., 2008). However, since two decades, significant increases in economic losses have also occurred in egg production at all ages of layer chickens (Zanella et al., 2000; Vandekerchove et al., 2004; Barnes et al., 2008). APEC is therefore a relevant disease in line with our aim to breed for increased general disease resistance.

For this study, we adopted a study model from broiler chickens to layer chickens, which has been described in several studies in relation to secondary APEC superinfections after primary viral infection (e.g. Matthijs et al., 2003; Dwars et al., 2009)). The used inoculated concentration of CFU/mL APEC is in line with previous studies on broilers (Matthijs et al., 2003; Dwars et al., 2009), though the actual volume inoculated is lower in this study. Broiler chickens suffering from colibacillosis can show: reduced activity, respiratory distress, growth

retardation, airsacculitis, diarrhea, lameness due to osteomyelitis, pericarditis, peritonitis, and yolk sac infection, amongst other pathological symptoms (Goren, 1978; Akashi et al., 1993; Dho-Moulin et al., 1999; Kunert Filho et al., 2015). Most of these pathological symptoms were observed in this study as well in a dose-response relation, suggesting that the severity of the infection might be dependent on the concentration of CFU/ml APEC in the inoculant. Overall, we conclude that the adoption of the broiler model to layer chickens was successful, and resulted in similar pathogenicity and clinical symptoms as for broiler chickens. The APEC model can therefore be used as an experimental model for investigation of APEC infection in layer chickens.

This study clearly shows that the High NAb selection line had a reduced mortality of 50% or more compared to the Low NAb selection line. Mortality was 9% for the High line and 24% for the Low line for $10^{8.20}$ CFU/mL APEC, 7% for the High line and 13% for the Low line for $10^{6.64}$ CFU/mL APEC, and 6% for the High line and 12% for the Low line for $10^{7.55}$ CFU/mL APEC. This line difference was significant in experiment 1. The aimed dose for the highest APEC group of experiment 2 was aimed to be equal to experiment 1, but unfortunately was 4.5 times lower compared to experiment 1. Mortality in experiment 2 is therefore lower than in experiment 1, and this most likely also affected the contrast between the High and Low line. This also illustrates the difficulty of using live diseases in infection experiments, which critically depend upon using the "optimum" infection dose in order to obtain the maximum contrast (and power) (Roederer, 2015). Nevertheless, the line differences observed for both concentrations of APEC in experiment 2 tended to significance, and confirmed that the High NAb selection line had a reduced mortality as compared to the Low NAb selection line.

The protective effect of NAb (selection) seems to have a dose-response (i.e. dose-survival) relation. The highest APEC concentration of the tested APEC doses had the best mortality distinctiveness (i.e. largest difference). Given the mortality in combination with different doses, and given theoretical models (for example described by Pessoa et al. (2014)), it can be expected that a higher APEC concentration (i.e. optimal dose) would give an even stronger distinctiveness for mortality. However for morbidity scores, a lower APEC concentration would be more distinctive: the optimal APEC dose for morbidity would probably be around the lowest APEC dose used in our experiments, because of the large diversity of observed scores. Determining an optimal dose for infection experiments is essential. This also means that a lack of significant line differences for mortality, or morbidity does not necessarily indicate no differences in resistance, but can be the result of the use of a non-optimal dose.

The dose-survival models also suggest that mortality occurs in the first period of the observational period (high dose), gradually during the observational period (medium dose), or at the end of the observation period (low dose), because the pathogen requires a certain concentration in the individual to cause mortality (Pessoa et al., 2014). One can speculate that the low APEC dose might have resulted in mortality differences, if the observational period was extended: i.e. the observational period is too short to observe mortality differences. For example, the $10^{6.64}$ CFU/mL APEC inoculated Control line showed equal

mortality to the High line, while both higher APEC doses caused significant differences in mortality. With an extended observational period, line difference in mortality might have become apparent. Especially because the $10^{6.64}$ CFU/mL APEC inoculated Control line had high morbidity scores compared to the High line, and chickens with high lesion scores might be prone to a higher mortality rate on short term (although this remains highly speculative). Nevertheless, this illustrates a major limitation of this experimental model: mortality after the experimental period would likely increase, and could go up to 100%. The actual survival, and more importantly the clearance of the APEC infection, and impact on the life (span) of the infected chickens cannot be estimated. Future studies should investigate optimal APEC doses for the highest distinctiveness on mortality, and morbidity, and should investigate the impact of APEC infection on the long-term.

The findings at post mortem (in the chicken that died during the experiment) was scored as sepsis, deposition of fibrinopurulent exudate in serosal surfaces of the lumina and air sacs (fibrin deposition), or both. Sepsis is injury to an individual's own tissues and organs as a result of the body's response to an infection, which can result in circulatory failure and eventually death (Singer et al., 2016). Septic shock is an acute state of sepsis (Singer et al., 2016), and likely occurred in the first 24-48 hours post inoculation (HPI). Fibrin deposition occurs at damaged locations in the body (Kuijper et al., 1997), and is therefore more likely the read-out of long-term damage due to APEC. Fibrin deposition occurred from 44 HPI onwards (experiment 1), or from 66 HPI onwards (experiment 2). In experiment 2, fewer chickens showed fibrin deposition at moment of death. It suggests that accumulation of APEC-induced fibrin deposition is time-, and dose-dependent. Therefore fewer chickens in the lower doses might have died yet with fibrin deposition, i.e. the observational time was too short to find similar observations. Ask et al. (2006b) hypothesized that the findings at post mortem (referred to as "cause of death") might reflect different APEC susceptibility (Ask et al., 2006b). However, no evidence for that is found in this study, because no line differences were observed for the findings at post mortem. It can therefore be suggested that APEC infection manifests regardless of disease resistance or susceptibility.

Morbidity, i.e. colibacillosis lesion scores, and especially variation in morbidity was mostly influenced by APEC dose, and hardly by line. In experiment 1, lesion scores were mostly absent for PBS inoculated chickens, and were mostly maximal for $10^{8.20}$ CFU/mL APEC inoculated chickens. Similar observations were done in experiment 2 for PBS inoculated chickens, and $10^{7.55}$ CFU/mL APEC inoculated chickens. Though the latter had slightly lower lesion scores, and slightly more variation. However the High line inoculated with $10^{6.64}$ CFU/mL APEC had significant lower lesion scores than the Control line, except for the pericardium although a similar trend was observed. The Low line was in between the High line and the Control line, and did not differ significantly. Systemic lesions were previously associated with increased susceptibility (compared to only airsacculitis) (Praharaj et al., 1996; Ask et al., 2006b). So even though the High line and the Control line had a similar mortality for $10^{6.64}$ CFU/mL APEC, the lesion scores suggest that the Control seems to be more affected by the APEC infection. It has to be kept in mind that morbidity was only

assessed on chickens that survived the experiment (i.e. higher APEC resistant chickens), and it might therefore be slightly biased. Therefore, lesions scores for assessing differences in disease resistance on population level should be interpreted with care.

The possible protective working mechanism(s) of (selective breeding for) NAb requires further investigation. Total KLH-binding NAb differences between High line and Low line both in generation 4 (experiment 1) and in generation 6 (experiment 2) were on average 2.1 titer points at 16 weeks of age (data not shown), which indicates no phenotypic increase of total KLH-binding NAb differences (selection criterion) between the selection lines from generation 4 to 6. However, phenotypic difference of KLH-binding IgM NAb and KLH-binding IgG NAb, and the estimated genetic difference (based on estimated breeding values (EBV)) increased between these generations (data not shown). It has to be kept in mind that selection for KLH-binding NAb occurred at 16 weeks of age, while the APEC model is used at 2 weeks of age. The level of maternal KLH-binding NAb in the offspring therefore may take a part in the protective effect of NAb, rather than the level of KLH-binding NAb of the individual itself. Regardless of the origin, KLH-binding NAb might possibly bind to APEC directly: Sundsmo et al. (2012) suggested that KLH contains *N*-glycans structurally similar to *E. coli N*-glycans (Sundsmo et al., 2012). As far as we know, this is the only direct link between KLH-binding NAb and APEC resistance.

APEC (or *E. coli*) was marked as amenable for (sufficient) genetic improvement of resistance (Davies et al., 2009). Our current NAb selection lines might add to better understanding of genetic difference in APEC resistance. Previous studies have reported varying levels of APEC-induced mortality, morbidity, or both in different chicken lines (Gross et al., 1980; Dunnington et al., 1991; Yunis et al., 2000, 2002a; Ask et al., 2006a; Calenge et al., 2013; Matthijs et al., in preparation). Others report large genetic variation for antibody response to *E. coli* vaccination within chicken lines (Pitcovski et al., 1987; Yonash et al., 1996; Cavero et al., 2009), and located APEC resistance-associated genomic regions on several chromosomes (Yonash et al., 2001; Yunis et al., 2002b; both taken from AnimalQTLdb, but see Hu et al. (2016b). Unpublished results show that a high APEC resistant broiler line has on average higher levels of KLH-binding NAb compared to a low APEC resistant broiler line (Matthijs et al., in preparation). However, Ask (2007) did not find a protective effect of NAb levels on susceptibility to colibacillosis in the study of Ask et al. (2006a), although the author did not specify when and what NAb types (antigen-binding, and isotype) were measured (Ask et al., 2006a; Ask, 2007). Taken together, genetic variation in APEC resistance is present, and studying the immunological differences in the NAb selection might add to better understanding of this genetic variation.

Poultry with a higher general disease resistance could be achieved by selective breeding for an improved disease resistance by using a populations natural genetic potential (Cheng et al., 2013). However, selective breeding for disease resistance based on challenge tests (i.e. infection experiments) is not feasible for implementation in selection programs, because these tests need to be performed on relatives, require large numbers of chickens, and severely affect animal welfare (Cavero et al., 2009). Instead, an indicator trait for disease resistance,

genomic regions underlying disease resistance, or both are required to improve disease resistance. The purpose of this study was to investigate NAb selection line differences on APEC-induced mortality, and morbidity. This study clearly shows that the High NAb selection line had a reduced mortality of 50% or more compared to the Low NAb selection line for the two highest APEC doses, but not for the lowest APEC dose. However, the High NAb selection line had reduced morbidity scores compared to the Low line. These results in combination with previous evidence (Star et al., 2007a; Sun et al., 2011; Wondmeneh et al., 2015) suggest that NAb might be a feasible strategy in breeding for improved general disease resistance in chicken. We therefore conclude that selective breeding for higher NAb levels increases APEC resistance, and possibly disease resistance in general. Future studies should expand to testing the NAb selection lines for differences in disease resistance for other types of diseases, like gram-positive bacteria, viruses, or protozoa.

Acknowledgement

We thank Lia Hemerik of Biometris, Wageningen University & Research for her statistical help in survival analyses. We thank Danny de Koning and Mariska Mesman for their help in statistical analyses and interpretation of the data. We also thank all involved animal caretakers of research facility “Carus”, and colleagues of Adaptation Physiology from Wageningen University & Research for their practical help before, during, and after the experimental period. This work is supported by Hendrix Genetics. This work is also part of the research programme ‘Divergent selection for *natural antibodies* in poultry’ with project number 12208, which is (partly) financed by the Netherlands Organisation for Scientific Research (NWO).

References

See List of references at the end of this thesis.

CHAPTER 7

General discussion

Introduction

High stocking density, and recent changes in production systems in modern poultry production increased the risk of diseases, and the spreading of pathogens within flocks (Atkins et al., 2013; Van Bunnik et al., 2014; Rozins et al., 2016). Diseases have a considerable impact on economic costs, and animal welfare (FAO, 2014). New, additional strategies to increase disease resistance are desired. One strategy is to increase general disease resistance through selective breeding.

Natural antibody (NAb) levels binding keyhole limpet hemocyanin (KLH) are associated with survival in layer chickens (Star et al., 2007a; Sun et al., 2011; Wondmeneh et al., 2015), and KLH-binding NAb have been shown to be heritable in chickens (Sun et al., 2013a; Sun et al., 2013b). NAb are defined as antibodies present in individuals without a previous exposure to these antigens (Vale et al., 2016). NAb have a wide range of protective effects in homeostasis, regulation of the immune system, and disease resistance (Boes, 2000; Zinkernagel, 2012; Born et al., 2016). Therefore selective breeding on KLH-binding NAb levels might increase general disease resistance.

Here, in the 'General discussion', I will discuss the results of this thesis in relation to the three objectives defined in the General Introduction (chapter 1):

1) to investigate the genetic variation of KLH-binding NAb levels in adolescent layer chickens (results of chapter 2, chapter 4, and this chapter);
2) to investigate the potential of KLH-binding NAb levels as an indicator trait for general disease resistance by
   a) divergently selective breeding on total KLH-binding NAb titers (this chapter), and
   b) inoculating these NAb selection lines with avian pathogenic *Escherichia coli* (APEC) (results of chapter 6, and this chapter); and
3) to investigate possible correlated responses of immune parameters, and production traits as a result of KLH-binding NAb selection (results of chapter 3, chapter 5, and this chapter).

In addition, I will suggest possibilities for future research to investigate questions left unanswered in my thesis, and I will formulate recommendations to industry.

Selective breeding for increased general disease resistance

Many (divergent) selective breeding experiments in chickens have been performed to improve general disease resistance using different indicator traits (see Terčič (2013) for a review). Some of these selection experiments showed promising effects on immune traits, and resistance to some diseases. However, except for elimination of certain MHC B-haplotypes, none of these indicator traits have been applied in breeding practices, as far as I know. This could be a result of, for example, the trait being poorly heritable, being too costly to measure, being too laborious to measure, being not indicative for multiple (i.e. general) disease resistance, or having undesirable correlated selection responses on

production traits. In addition, industry might have been reluctant to apply the strategy, because, for example, selection on survival is already part of the selection index. In general, it can be stated that selection experiments did not have the anticipated impact for application in industry (yet).

What makes selective breeding for total KLH-binding NAb titers at 16 weeks of age different from previous selection experiments, is that NAb have a broad working range, have been associated with a relevant trait (i.e. survival) in several chicken lines, are easy, and relatively cheap to measure in large quantities, do not require (pre-)treatment of the individual, and the necessary blood sample is already collected for genotyping in the current management strategy in poultry breeding. Thus, selective breeding for KLH-binding NAb titers can be relatively easily implemented. In addition, measuring NAb levels in individuals might give predictive information on general disease resistance, in contrast to measuring only the outcome of differences in general disease resistance (e.g. mortality).

Selective breeding for total KLH-binding NAb titers at 16 weeks of age

The selection trait in this selection experiment was total KLH-binding NAb titers (based on heavy, and light chain-binding ELISA) at 16 weeks of age. Mass selection was applied, meaning that individuals were selected only on their own performance. The selection experiment was started from the base population described in chapter 2, and was continued for six generations in total. The set-up of the selection experiment is described in chapter 4, and chapter 5. An overview on the set-up of the selection experiment, settings for SelAction for additional analyses, and used statistical models for additional genetic analyses can be found in Box 1.

Selection response based on NAb titers

After six generations of selection, a clear, and significant phenotypic difference in total KLH-binding NAb (IgT) titers[1] is observed between the High line, and the Low line (Figure 1): IgT differed 2.0 titer points, which means that the High line has on average roughly a ($2^{2.0}$=) 4.0 times higher IgT level compared to the Low line. Also the correlated selection responses of KLH-binding IgM titers (2.7 titer points difference), and KLH-binding IgG titers (2.3 titer points difference) significantly differed between the High line, and the Low line, as was expected based on the high genetic correlations between the different NAb types (chapter 2). It can therefore be concluded that divergent selective breeding for total KLH-binding NAb titers is possible, and that simultaneous improvement for different KLH-binding NAb types is possible as well.

[1] I would like to thank Joop A.J. Arts (Adaptation Physiology, Department of Animal Sciences, Wageningen University & Research) for measuring all described traits in the 'General discussion' unless stated otherwise, and valuable discussion.

Box 1. Details on the selection experiment

Set-up of the selection experiment

Base population

The base population, not selected previously on NAb, or related traits, consisted of almost 3,700 commercial purebred White Leghorn chickens (approximately 2,400 females, and 1,300 males) from the "WA" line of "Hendrix Genetics". This layer chicken line has been mainly selected for egg production, but also other production traits, e.g. traits related to egg quality. The chickens were kept according to standard management of breeding nucleus farms of Hendrix Genetics.
Plasma of the studied chicken population was collected at 15 weeks of age (males), or 19 weeks of age (females), and stored at −20°C until use. These plasma samples were used to measure the selection criterion: total KLH-binding NAb titer in a 4-step dilution ELISA (for measurement: see chapters 2-5). 25 males, and 50 females with the highest titers were selected to breed generation 1 of the High line. 25 males, and 50 females with the lowest titers were selected to breed generation 1 of the Low line.

NAb selection lines

Incubation of eggs, and housing of chickens for the selection lines (from generation 1 onwards) was at research facility "Carus" from Wageningen University & Research according to standard production practices. However, per generation some practices might differ, depending on updated management practices, or temporary conditions.
Each generation consisted of approximately 600 chickens per line. The two lines were mixed within sex, and were housed within one stable, or per sex in two adjacent stables. Plasma samples were collected at 16 weeks of age, and stored at −20°C until use. These plasma samples were used to measure the selection criterion: total KLH-binding NAb titers at 16 weeks of age in a 4-step dilution ELISA, and a 8-step dilution ELISA (for measurement: see chapters 2-5). Similar to the base population, 25 males and 50 females within a line were selected to breed the next generation of that line.

Settings for SelAction for additional analyses

SelAction v2.2 (Rutten et al., 2002) was used to predict selection responses for total KLH-binding NAb (IgT) titers, or KLH-binding NAb isotype titers (IgM, IgA, and IgG) based on the base population. The settings can be found in Table A (general settings), Table B (heritability settings), and Table C (correlation settings).

Box 1 (continued)

Table A. General settings in SelAction to estimate selection responses for total KLH-binding NAb (IgT) titers, or KLH-binding NAb isotype titers (IgM, IgA, and IgG) based on the base population.

Settings	Discrete Generations; 1 - stage selection
# Selected parents	25 males, 50 females
# Offspring per dam	5 males, 5 females
Proportion selected parents	25/250 males, 50/250 females
Index	Own performance for IgT only

Table B. Heritability settings in SelAction to estimate selection responses for total KLH-binding NAb (IgT) titers, or KLH-binding NAb isotype titers (IgM, IgA, and IgG) based on the base population. Shown per NAb (iso)type is the number of observations (n), the phenotypic variance (σ_p^2), the heritability (h^2), and the maternal environmental effect (m^2) (taken from Chapter 2). The maternal environmental effect was set as common environment variance (c^2) in SelAction.

	IgT	IgM	IgA	IgG
n	3,689	3,689	3,547	3,689
σ_p^2	1.86	1.27	1.50	1.68
h^2	0.12	0.14	0.10	0.07
m^2	NS	0.06	NS	NS

Table C. Correlation settings in SelAction to estimate selection responses for total KLH-binding NAb (IgT) titers, or KLH-binding NAb isotype titers (IgM, IgA, and IgG) based on the base population. Estimated genetic correlations are shown below the diagonal, and phenotypic correlations are shown above the diagonal (taken from Chapter 2).

	IgT	IgM	IgA	IgG
IgT	-	0.55	0.30	0.81
IgM	0.97	-	0.33	0.26
IgA	0.92	0.81	-	0.22
IgG	0.96	0.86	0.87	-

Statistical models for additional genetic analyses

The statistical models for estimating genetic parameters of total KLH-binding NAb (IgT) titers, or KLH-binding NAb isotype titers (IgM, IgA, and IgG) is described in chapter 2. For estimation of variance components of IgT, IgM, IgA, and IgG NAb titers within the base population, a linear animal model was used, including a fixed plate effect, a random additive genetic effect, and, in case of a significant maternal environmental effect (only IgM), a random dam effect. A pedigree was used, consisting of 4,586 individuals, and was based on 7 generations (see chapter 2).

For estimation of variance components of IgT, IgM, and IgG NAb titers within the NAb selection lines (including the base population), a linear animal model was used, including a fixed plate effect, a fixed sex effect, a random additive genetic effect, and a random dam effect. A pedigree was used consisting of 9,712 individuals, and was based on 13 generations.

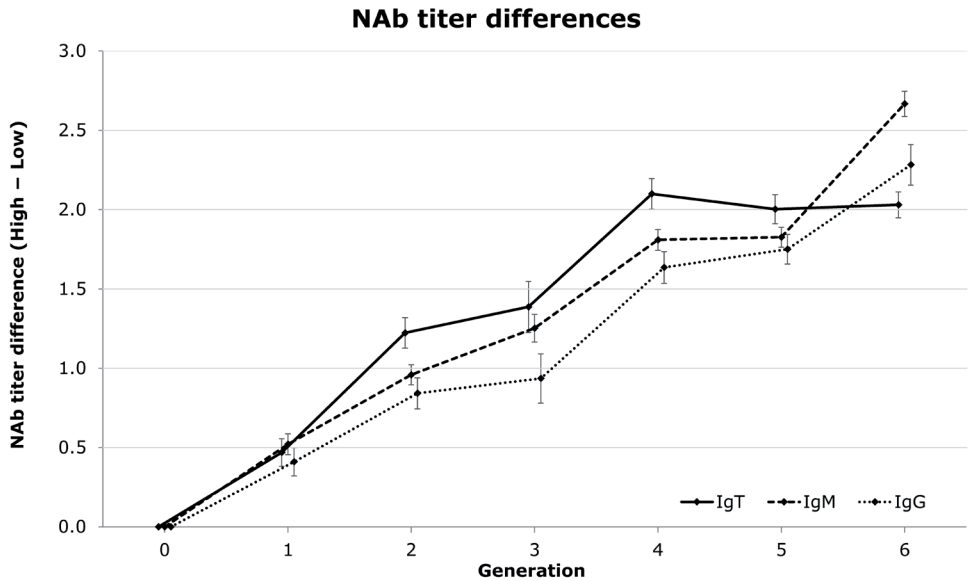


Figure 1. Least square (LS) phenotypic line differences (and SE) of NAb titer (High line – Low line) over six generations of divergent selective breeding for total KLH-binding NAb titers (IgT) at 16 weeks of age (solid line). In addition, the correlated responses of LS phenotypic line differences of KLH-binding IgM titers (dashed line), and KLH-binding IgG titers (dotted line) are shown. General linear models were used to statistically test for line differences: all NAb (iso)type titers in all generations were significantly different between the High line, and the Low line.

Heritabilities were determined to estimate the genetic variation of KLH-binding NAb titers in the base population (chapter 2, and Box 1): the heritabilities were: 0.12 for IgT, and 0.14 for IgM, 0.10 for IgA, and 0.07 for IgG, which are in line with most heritabilities reported for immune(-related) traits.

These estimates, and the described set-up of the selection experiment (Box 1) can be used to predict the selection response before the start of the selection experiment (SelAction v2.2; Rutten et al. (2002)). The predicted selection responses in titer difference per generation were: 0.50 for IgT, 0.43 for IgM, and 0.35 for IgG (and 0.38 for IgA). The average realized increases in titer point differences per generation were: 0.36 for IgT, 0.41 for IgM, and 0.37 for IgG (based on the slopes of the fitted trendlines in Figure 1, data not shown). Up to generation 4, the average realized increase was 0.51 for IgT, but from generation 4 to generation 6, no increase in titer difference was observed. This irregular response ("waves of response"; Dunnington et al. (1996)) has been reported in other selection experiments as well. Reasons underlying possible stagnation in selection responses will be discussed later. The stagnation in selection response was not observed for the IgM, or IgG: the selection responses were similar to the predicted responses.

Re-estimation of genetic parameters

The 5,101 NAb observations on chickens of the selection experiment, and the 3,689 observations of the base population allowed for more accurate (re-)estimation of genetic parameters of KLH-binding IgT, IgM, and IgG NAb titers (Table 1)[2]. Heritabilities were 0.11 for IgT, 0.27 for IgM, and 0.11 for IgG. The heritabilities for IgT, and IgG were almost equal to the previously estimated heritabilities in the base population (chapter 2). Remarkably, the heritability of IgM NAb was twice as high as estimated before. In chapter 2, a heritability of 0.29 was reported when maternal environmental effects were not taken into account. Here, a heritability of 0.30 was estimated without maternal environmental effect. However, with increasing maternal environment effects (0.06, 0.10, and 0.15), IgM heritability was unaffected (0.26 for all). This suggests that the maternal effect did not bias the estimate. This also suggests that the previous estimate in the base population of IgM NAb (chapter 2) might have been underestimated. Though it should be kept in mind that a heritability estimate is not only a property of the trait, but also of the investigated population: the heritability is influenced by both the genetic component of a population (i.e. allele frequencies), and the residual (i.e. mainly environmental) component of a population (e.g. management conditions) (Falconer et al., 1975). For the re-estimated IgM heritability, the environmental variance was smaller compared to the estimates in chapter 2 (0.78 vs. 1.02), possibly due to standardized housing, and management conditions for the NAb selection lines. In addition, the additive genetic variance was larger (0.31 vs. 0.18). The heritabilities are more in line with the studies of Sun et al. (2013a), and Sun et al. (2013b), although these studies did/could not take maternal effects into account. Some bias in estimating the IgM heritability might have occurred due to the associated genomic region on chromosome 4 (chapter 4) with a large effect, full dominance, and changed allele frequency in the NAb selection lines. The applied analyses do not account for changes in allele frequency, and assume the infinitesimal model (assuming that a trait is influenced by many genes with each a small effect). Also the additive genetic variance does not include dominance variation. Although in general, the

Table 1. Heritability (re-)estimates for total KLH-binding NAb (IgT) titers, or KLH-binding NAb isotype titers (IgM, and IgG) based on the base population, and the NAb selection lines (n = 8,790). Shown per NAb (iso)type is the number of observations (n), the phenotypic variance (σ_p^2), the heritability (h^2), and the maternal environmental effect (m^2). IgA was not measured in the selection experiment, and is therefore not included.

	IgT	IgM	IgG
σ_p^2	1.92	1.13	2.03
h^2	0.11	0.27	0.11
m^2	0.03	0.04	0.02

[2] These results are partly based on the MSc thesis of Komarudin (Wageningen University & Research), supervised by Henk Bovenhuis (Animal Breeding and Genomics, Department of Animal Sciences, Wageningen University & Research), and myself.

performed analyses are known to be rather robust. Re-estimation with observations on the base population, and the selection lines suggests that more genetic variation might be present in the population for IgM NAb compared to the base population.

Selection response based on EBV

Re-estimation of genetic parameters also allowed investigation of the realized selection response based on estimated breeding values (EBV; the genetic potential of an individual compared to the average of the population). The realized selection responses based on EBV should be close to the realized selection responses based on titers (Figure 1), when environmental conditions have not changed in time. Indeed, the realized selection responses for IgT, and IgG (Figure 2) were comparable (based on the slopes of the fitted trendlines in Figure 2, data not shown): the selection response based on EBV for IgT was 0.36 (0.18 per selection line) per generation, and the selection response based on EBV for IgG was 0.32 (0.16 per selection line) per generation. Interestingly, the line difference in EBV for IgT increased from generation 4 to generation 6, although no increase in phenotypic line difference was observed. This suggests that genetic progress for IgT due to selection was still made, though not phenotypically observed.

The realized selection response based on EBV for IgM (Figure 2) was also similar to the

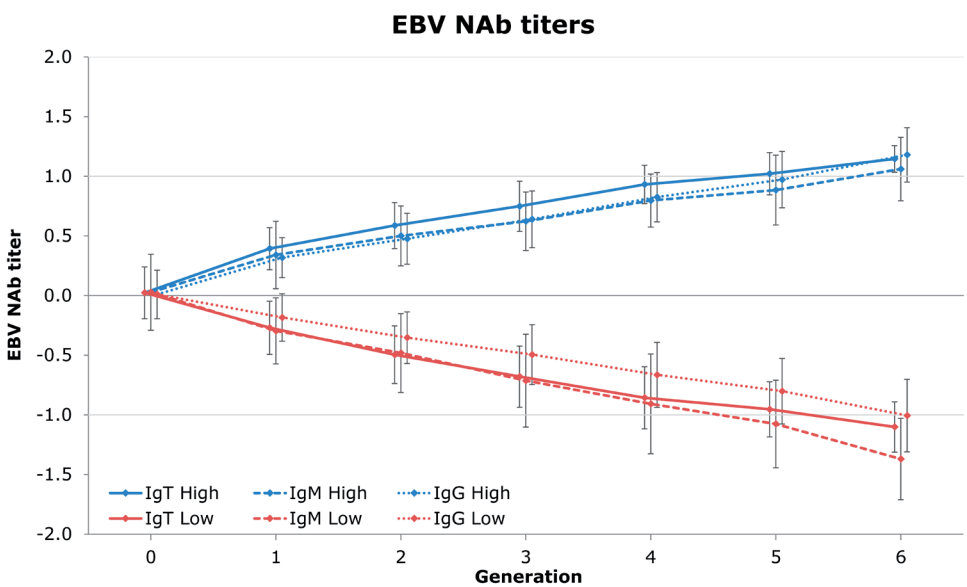


Figure 2. Average estimated breeding values (EBV) (and SD) of NAb titers over six generations of divergent selective breeding for total KLH-binding NAb titers (IgT) at 16 weeks of age (solid line). In addition, the correlated responses of KLH-binding IgM titers (dashed line), and KLH-binding IgG titers (dotted line) are shown. The High line is shown in blue, and the Low line is shown in red. Box 1 describes the used statistically model to estimate EBV per line, and per generation.

realized selection response based on titers (Figure 1): 0.40 per generation. However, the realized selection response in the High line was slightly lower compared to the Low line: 0.18 vs. 0.22, which creates an EBV-based difference of 0.24 titer points after six generations. Asymmetric responses ("waves of response"; Dunnington et al. (1996)) can (theoretically) be a result of random drift, different selection differentials, inbreeding depression, indirect selection, genetic asymmetry, scalar asymmetry, and genes with large effects (Falconer et al., 1975). Effects of random drift can only be quantified when the selection experiment is repeated. Different selection differentials may dependent on natural selection (e.g. line differences in fertility, or mortality) favoring a selection direction, or may dependent on variances depending on the mean. Inbreeding depression has likely not accumulated to such extent that it has detrimental effects: the estimated inbreeding coefficient per generation (based on SelAction) was below the recommended 1%. Indirect selection is the result of confounded effects. Some possible indirect effects could be antibody characteristics other than quantity (e.g. antibody affinity), but these effects are difficult to identify. Some minor scalar asymmetry seemed to occur during the selection for the Low line: for example, the standard deviation of the EBV in generation 1 was 0.28 for both selection lines, and in generation 6 was 0.27 for the High line, and 0.34 for the Low line. In addition, a small right tail skewness was observed for EBV of the Low line. These results suggest that the selection response for the Low line is lower. However, the realized selection response based on EBV of the Low line seemed unaffected, but the realized selection response based on EBV of the High line decreased. This is most likely the result of a combined effect of genetic asymmetry, and genes with large effects, which will be explained in the next section.

Association of *TLR1A*

A genomic region on chromosome 4 was associated with KLH-binding IgM NAb titers (chapter 4). The genomic region contains one single nucleotide polymorphism (SNP) in a coding region of Toll-like receptor 1A (*TLR1A*) (Box 2) with a high predicted impact on protein folding, and functioning. This SNP (C/G polymorphism) showed a dominant mode of gene action (for the C-variant), which was associated with higher IgM NAb titers. The SNP explained 63.5% of the additive genetic variation (chapter 4). For the remainder of the 'General discussion', I assume the SNP in *TLR1A* is the causal variant underlying the observed differences.

The average effect of the *TLR1A* variant substitution (α) can be estimated by formula [7.5] of Falconer et al. (1975):

$$\alpha = a + d(q - p)$$

where a is the additive effect of the variant substitution ($\frac{1}{2} \times (effect_{CC} - effect_{GG})$), d is the dominance effect of the variant substitution ($effect_{CG} - \frac{1}{2} \times (effect_{CC} + effect_{GG})$), q is the G-variant frequency, and p is the C-variant frequency. The genotype class effects were based on the GWAS results: 0.04 for CC, 0 for CG, and −0.61 for GG (Table 5,

Box 2. Toll-like receptors (TLR)

TLR are among the most studied immune receptors. **TLR are a family of transmembrane proteins that recognize conserved molecular patterns (microbe-associated molecular patterns (MAMP)/danger-associated molecular patterns (DAMP)).** TLR are highly conserved in evolution, and their existence can be dated back to the dawn of animal evolution (Leulier et al., 2008). All TLR have similar structures, consisting of an array of leucine-rich repeats (LRR) motifs, a transmembrane domain, and cytoplasmic Toll/IL-1 receptor domain (Keestra et al., 2007).
Because of their presence in lower vertebrates, it is likely that these receptors originated from the innate immune system, and many innate functions of TLR have been described. However, it is well known now that these receptors also play important roles in the adaptive immune system (e.g. Bekeredjian-Ding et al. (2009)).
For a review on avian TLR, see Brownlie et al. (2011).

chapter 4), and the *TLR1A* variant frequencies were 0.45 for the C-variant, and 0.55 for G-variant. This results in: a = 0.325, d = 0.285, q = 0.55, p = 0.45, and α = 0.3535. The additive genetic standard deviation (σ_a) over the *TLR1A* polymorphisms can calculated for the GWAS population (assumed to be equal for the selection experiment) by taking the square root of formula [8.5] of Falconer et al. (1975):

$$\sigma_a = \sqrt{2pq\alpha^2}.$$

The variant frequencies can be varied to estimate the genetic standard deviation based on *TLR1A* variants (Figure 3). Important to realize is that the genetic standard deviation is directly related to the selection response ($R = ih\sigma_a$, with i being the selection intensity), and to the heritability ($h^2 = \frac{\sigma_a^2}{\sigma_p^2}$).

To investigate segregation of the *TLR1A* variants in generation 6, all female chickens were genotyped for the *TLR1A* variants[3]. In total, 187 High line females, 81 Control line females, and 170 Low line females were genotyped. The *TLR1A* variant frequency in the High line was 0.64 for the C-variant, and 0.36 for the G-variant, in the Control line 0.47 for the C-variant, and 0.53 for the G-variant, and in the Low line 0.08 for the C-variant, and 0.92 for the G-variant. For the Control line, no change in allele frequencies was observed, which suggests no selective pressure, or random genetic drift on the *TLR1A* variants. Variant frequency changes were observed, as can be expected based on estimated effects (chapter 4): the C-variant is favorable for the High line, and the G-variant is favorable for the Low line. However, the chickens were selected on (IgT) phenotype (and not *TLR1A* variant). Therefore it is difficult to fix the C-variant in the High line, because of the dominant mode of action

[3] I would like to thank Marleen H.P.W. Visker (Animal Breeding and Genomics, Department of Animal Sciences, Wageningen University & Research) for determining the *TLR1A* variants.

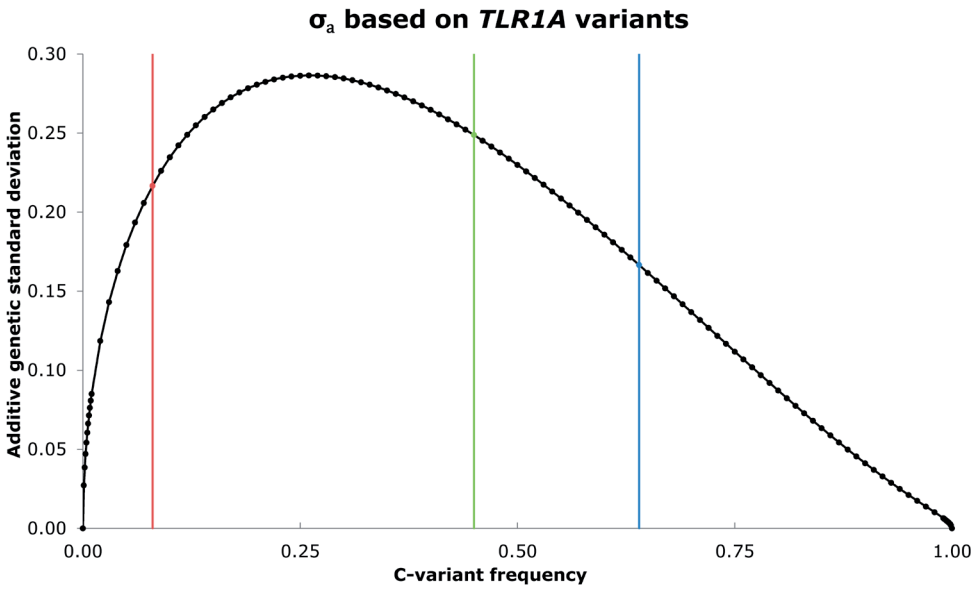


Figure 3. Estimated genetic standard deviation based on TLR1A C-variant frequency. The genotype class effects were based on the GWAS results: 0.04 for CC, 0 for CG, and −0.61 for GG (Table 5, chapter 4). The red vertical line indicates the C-variant frequency in generation 6 females of the Low line (0.08), the green vertical line indicates the C-variant frequency in the GWAS population (0.45), the blue vertical line indicates the C-variant frequency in generation 6 females of the High line (0.64).

(no phenotypic discrimination between CC genotype class, and CG genotype class). While the G-variant is relatively easy to fix in the Low line, because GG is phenotypical different from CC genotype, and CG genotype. This can also be seen in Figure 3, which shows the additive genetic standard deviation as a function of the C-variant frequency in the GWAS population (0.45), and the additive genetic variation for the C-variant frequencies in the NAb selection lines (0.64 for the High line; 0.08 for the Low line). A reduction in additive genetic variation is observed, when selecting for the C-variant for the High line. While an increase in additive genetic variation is observed, when selecting against the C-variant for the Low line (i.e. select for the G-variant). It means that from the starting position in the base population, the selection response is expected to increase when selecting for higher frequencies of the G-variant. A higher frequency of the G-variant means that the genetic standard deviation will increase, and consequentially the selection response ($R = ih\sigma_a$). At the current stage of selection (i.e. *TLR1A* variant frequencies in generation 6 females) in the NAb selection lines, it can be expected that the High line will have a lower selection response due to smaller additive genetic variation compared to the Low line. Indeed, IgM heritability estimates based on generation 5, and generation 6 only, showed a reduced heritability of 0.18 for the High line, but an unaffected heritability of 0.26 for the Low line (both with the maternal environmental effect fixed to 0.04). So the reduced genetic selection response of the High line can be explained, as stated in the previous section: “[t]his is probably the result

Table 2. Least square means (and SE) of KLH-binding IgT, IgM, and IgG NAb titers at 16 weeks of age of the *TLR1A*-variant (C/G) genotyped females within a line (High line, Control line, or Low line) of generation 6. A general linear model was used to statistically test for line differences, which are indicated with different superscript letters on a row ([a]-[f]). The CC genotype class of the Low line contained only 3 chickens, which resulted in large SE.

	High line (n = 187)				Control line (n = 81)				Low line (n = 170)			
	CC (SE)		CG (SE)	GG (SE)	CC (SE)		CG (SE)	GG (SE)	CC (SE)		CG (SE)	GG (SE)
n	75		88	24	18		40	23	3		22	145
IgT	7.8^{a}	(0.1)	7.7^{a} (0.1)	7.7^{a} (0.2)	7.4^{a}	(0.3)	7.3^{a} (0.2)	6.8^{b} (0.2)	5.3^{c}	(0.7)	5.9^{c} (0.3)	5.9^{c} (0.1)
IgM	6.3^{ab} (0.1)		6.5^{a} (0.1)	5.8^{c} (0.2)	6.3^{abc} (0.3)		5.9^{bc} (0.2)	5.1^{d} (0.2)	3.6^{ef}	(0.6)	4.1^{e} (0.2)	3.6^{f} (0.1)
IgG	6.8^{a} (0.2)		6.7^{ab} (0.2)	7.0^{a} (0.4)	6.4^{abc} (0.4)		6.4^{ab} (0.3)	5.9^{b} (0.4)	4.1^{c}	(1.1)	4.6^{c} (0.4)	4.5^{c} (0.2)

of a combined effect of genetic asymmetry [(i.e. full dominance)], and genes with large effects [(i.e. *TLR1A*)]".

The *TLR1A* genotyped females of generation 6 also allowed confirmation of NAb titer differences at 16 weeks of age between genotype classes within a line (Table 2). The differences between genotype classes were more or less equal to the estimated effects in the GWAS (0.04 for CC, 0 for CG, and −0.61 for GG (Table 5, chapter 4)). The CC genotype, and CG genotype were significantly higher than then GG genotype class for the High line, and the Control line. But was not significantly different for the Low line: the CC genotype class was equal to the GG genotypes class, because the CC genotype class consisted of 3 chickens, and therefore standard errors of the average were large. The observed genotype class differences confirm that the *TLR1A* variants is associated with KLH-binding IgM NAb titers. The observed genotype class differences of the Control line also confirm the associations between *TLR1A* variants, and KLH-binding IgT and IgG titers in the unselected (Control) line (Table 5, chapter 4). Fixation of the C-variant in the High line, and the G-variant in the Low line has occurred for 40% of the High line chickens, and 85% of the Low line chickens. Fixation of the C-variant in the High line, and the G-variant in the Low line can lead to a maximum phenotypic line difference of 0.65 titer point. The average KLH-binding IgM NAb titer based on the frequency of *TLR1A* genotype classes, and the predicted effects (0.04 for CC, 0 for CG, and −0.61 for GG (Table 5, chapter 4)), is 0.55 in the High line, and 0.09 in the Low line. The current phenotypic line difference has reached 70% of the maximum phenotypic line difference, and an increase of 30% in phenotypes between lines is still possible (based on fixation of the *TLR1A* variants). However, phenotypic selection on IgT will not (necessarily) lead to fixation, because IgT phenotypes were not different between genotype classes within the selection lines of the generation 6 females (Table 2). Fixation of both variants in the NAb selection lines might therefore only be reached by direct selection based on SNP information.

Concluding remarks

To conclude this section, divergent selective breeding for total KLH-binding NAb titers at 16 weeks of age can be successfully applied, as is illustrated in the NAb selection experiment. The high genetic correlations between different NAb (iso)types suggests that IgT indeed reflects all three isotypes, and that therefore simultaneous improvement of all NAb isotypes is possible.

A lower selection response was realized in the phenotypic IgT line differences for the last two generations, but EBV suggested that genetic improvement has still been made. On the other hand, EBV for IgM suggested that additive genetic variation in the High line was reduced. In the long term, a plateau for NAb levels will likely occur (selection limit (Falconer et al., 1975)), of which the first signs might have been observed for high KLH-binding IgM NAb titers. A plateau for low levels of NAb was not observed yet, but might be expected to establish after more generation due to a lack of genetic variance, detection limits, or opposing forces (Falconer et al., 1975). Detection limits were observed for the sheep red blood cell (SRBC) immunization-selection experiment (described by Bovenhuis et al. (2002)). Although the KLH-binding ELISA procedure is more sensitive compared to SRBC agglutination. Opposing forces might be a particular risk for the Low line, if NAb are indeed associated to survival/general disease resistance: natural selection will eliminate individuals with too low levels of NAb from the population before they can be used for breeding purposes. Continuation of the NAb selection experiment will provide answers to these questions.

Underlying physiological differences between the NAb selection lines, causing differences in NAb titers, should be a main focus point of future research. In chapter 4, IgM NAb titers were shown to be highly genetically correlated to total IgM concentrations. Other correlated selection responses will be discussed later in this thesis. But a good initial strategy to investigate possibly physiological differences, is to frequently measure NAb titers, and antibody concentrations. In addition, sacrificing some birds at these moments to investigate immunological organ weights (especially bursa of Fabricius (Box 3), and spleen), cell isolation, and histochemistry might give insight on physiological differences, and underlying causes of different NAb levels.

Future research should also focus on confirming the causal mutation of *TLR1A*, and its effect on antibodies. A first step could be to transfect, for example, HeLa-cells with the two *TLR1A* variants, and stimulation with known TLR1/2/6 ligands (e.g. peptidoglycan, lipoteichoic acid, Pam_3CSK_4) in combination with TLR2A/B, and possibly associated coreceptors. A second step could be to isolate (different) cell populations from the blood, or the spleen, and investigate their responses (cytokine production/expression, and expression of TLR, and downstream products) to different stimuli. A third step could be to investigate the physiological differences of the *TLR1A* variants (within lines) in relation to vaccination, or different feed strategies.

Box 3: The bursa of Fabricius, and B cell ontogeny

Both NAb, and SpAb are produced by B cells. In chickens, B cells are produced in the bursa of Fabricius, which also gave name to the B cell. The bursa is a primary lymphoid organ present at hatch, and reaches its maximum seize around 8 weeks of age. Involution of the bursa starts around 10 weeks of age as a result of sexual maturation, excels around 20 weeks of age, and ends around 28 weeks of age when the bursa has almost completely disappeared (Ciriaco et al., 2003; Oláh et al., 2014). Thus, in contrast to B cell production in the bone marrow of some mammals, chickens have only a limited time frame of B cell development, and B cell production.

The bursa is located between the cloaca, and the sacrum, and is directly connected to the gut by the bursal duct (Oláh et al., 2014). The bursa facilitates direct contact between gut-derived material (e.g. microbiota, or MAMP), and developing B cells. Although gut-derived material is not essential for B cell development, exposure to gut-derived material boosts B cell maturation, and B cell proliferation (Ekino et al., 1979; Ratcliffe et al., 2014), increases bursal weight, and immune reactivity (Ekino et al., 1980), and is therefore of major influence on B cell ontogeny.

Additional to future research of the genetic variants of *TLR1A*, genotyping the NAb selection lines might identify new genomic regions. This is aided by the larger study population, but mainly by changes in gene frequencies between the NAb selection lines ("signature of selection"; e.g. Bamshad et al. (2003)).

Finally, further investigation of maternal effects on NAb levels might be an interesting research area as well. In general these maternal effects are seen as a nuisance in quantitative genetics, but these effects might be utilized in such a way that beneficial maternal effects are transferred to crossbred offspring to increase disease resistance in commercial settings. It is also tempting to speculate that the observed maternal environmental effects might actually have a genetic component (i.e. maternal genetic effect). A maternal genetic effect (possibly because of *TLR1A*) could not significantly be detected in the used populations, but should not be ruled out. Maternal genetic effects are difficult to detect, because the genotype of the offspring are partly confounded with the genotype of the mother. In addition, the mother might not produce offspring with all genotype classes. However, the relevance for future research on selection for NAb levels also depends on its possible protective contribution to general disease resistance. This will be discussed in the next section.

Selective breeding on natural antibodies and general disease resistance

To test if divergent selective breeding for total KLH-binding NAb titers also resulted in differences in disease resistance, the NAb selection lines were inoculated with avian pathogenic *Escherichia coli* (APEC) at young age. APEC is a gram-negative bacterium,

which can cause colibacillosis, and eventually death. APEC is an opportunistic pathogen: APEC is present in the environment (i.e. gastrointestinal tract) of the chicken, but is capable of infecting the chicken (often via the respiratory tract) if the opportunity arises (Dziva et al., 2008; Ewers et al., 2009; Guabiraba et al., 2015). APEC has resistance against several antibiotics, and vaccination with heterologous strains is not sufficiently protective (Dho-Moulin et al., 1999; Kariyawasam et al., 2004; Sadeyen et al., 2014). APEC was identified as one of the sixteen poultry diseases amenable for genetic improvement of resistance, based on scoring for industry concern, economic impact, public concern, threat to food safety/zoonotic potential, impact on animal welfare, and threat to international trade barriers (by Davies et al. (2009)).

Previous studies showed NAb levels to be associated with mortality (Star et al., 2007a; Sun et al., 2011; Wondmeneh et al., 2015). Albeit the mortality cause was not defined in these studies, the cause of mortality is likely not APEC (only). However, the main focus of the infection experiments was on differences in mortality due to a defined cause (chapter 6). The High line had significant lower mortality compared to the Low line, but the protective effect was less pronounced when the APEC inocula were lower in concentration. Morbidity scores of colibacillosis were significantly lower, or tended to be significantly lower in the High line compared to the Low line. In addition, the body weight gain during infection, weight of the bursa, liver, and spleen were in general significantly higher in the High line compared to the Low line (all 'corrected' for body weight, by including body weight as a covariate in the model). This strongly suggests that the High line suffered less from APEC (with same starting dose) at young age compared to the Low line. Overall, it was concluded that selection for higher total KLH-binding NAb titers facilitates APEC resistance.

The infection experiments were performed in two different generations. The increase between generation 4, and generation 6 for genetic (i.e. EBV) line differences at 16 weeks of age were (Table 3): 0.38 for IgT EBV, 0.41 for IgM EBV, and 0.37 for IgG EBV (based on averages of the NAb selection lines in the selection experiment) (Table 3). This suggests that the difference in APEC resistance due to NAb selection can be expected to be more pronounced in generation 6 compared to generation 4. However, because of (unintentional) different APEC doses used between the experiments, a direct comparison is not possible between different doses in the NAb selection lines.

The protective effect of NAb (selection) seems to have a dose-response (i.e. dose-survival) relation: the highest APEC concentration had the best mortality distinctiveness (i.e. largest

Table 3. The genetic line differences (based on estimated breeding values; EBV) between the High line, and the Low line of generation 4, and generation 6, and the increase of line differences between generation 4, and generation 6.

	IgT	IgM	IgG
Generation 4 (High – Low)	1.79	1.71	1.48
Generation 6 (High – Low)	2.25	2.43	2.19
Increase (gen. 6 – gen. 4)	0.38	0.41	0.37

difference) of the tested APEC doses. Given the mortality in combination with different doses, it can be expected that a higher APEC concentration would give an even stronger distinctiveness (i.e. optimal dose), according to Pessoa et al. (2014). A higher mortality was observed in a pilot experiment, performed to determine the desired dose for the infection experiments. One of the investigated APEC doses was approximately five times higher than the highest dose used in the described experiment, and had a mortality of 73% (11/15 inoculated Control chickens), which is the inverse of the highest APEC dose used in the infection experiments. However, the NAb selection lines were not tested with this dose, and no definite conclusions can be drawn. For morbidity scores, a lower APEC concentration would be more distinctive: the optimal APEC dose for morbidity would probably be around the lowest APEC dose used in our experiments, because of the large diversity of observed scores. Determining an optimal dose for infection experiments is essential. This also means that a lack of significant line differences for mortality, or morbidity does not necessarily indicate no differences in resistance, but can be the result of a suboptimal dose.

Of particular interests is that not only selection on KLH-binding NAb levels had a protective effect, the selection had a protective effect at an age before the selection criterion. It suggests that NAb levels around adolescence are associated with survival during laying period (Star et al., 2007a; Sun et al., 2011; Wondmeneh et al., 2015), and that NAb selection around adolescence also had an effect on early life survival as well. Remarkably, the NAb selection had even an effect before the immune system of the individual itself has fully matured. This might be a result of transgenerational parental effects, most likely of maternal origin. Maternal (natural) antibodies might provide protection, although their role remains to be elucidated: no protective effects on mortality was found for KLH-binding NAb levels, and APEC-binding (N)Ab levels (as covariates) in blood of siblings (0 days old) of the infected chickens, or in blood of the mothers (taken directly after ending the egg collection period) (data not shown). In addition, the Control line had NAb titers that were closer to the High line than to Low line in the first experiment (data not shown), but mortality was equal to the Low line. Also Ask (2007) reported no protective effects of maternal antibody/NAb levels on colibacillosis in broilers in a similar experimental set-up (Ask, 2007), and these seem therefore not to be a major importance to disease resistance.

Possible differences in APEC resistance, in this relatively short-time experiment, might be distinguished in innate immunity differences, and protective effects of NAb level (selection) might not have been apparent yet. Several transcriptomic papers of the Lamont-group indicate a major role of innate immunity on APEC resistance/susceptibility: for example, TLR, and TLR pathway expressions were significantly different in (relatively) resistant chickens compared to susceptible chickens in the spleen, blood leukocytes, bone marrow, and the thymus (Sandford et al., 2011; Sandford et al., 2012; Sun et al., 2015a; Sun et al., 2016a; Sun et al., 2016b), except for the bursa (Sun et al., 2015b). The associated *TLR1A* variants present in the NAb selection lines can be of major influence on APEC resistance in our selection lines. The *TLR1A* variants can influence resistance in both a positive way (APEC sensitivity), or a negative way (lack of sensitivity, or oversensitivity). In the

Lamont-papers, TLR1A expression was increased in the bone marrow (Sun et al., 2015a), and in the thymus of APEC susceptible chickens (Sun et al., 2016b). It has to be mentioned that TLR1A recognizes peptidoglycans, amongst other ligands, which are more present, and more easily accessible for binding on gram-positive bacteria compared to gram-negative bacteria. TLR1A is therefore often associated with gram-positive bacterial immunity, while *E. coli* is a gram-negative bacterium. Nevertheless, future research using genotypes of the NAb selection chickens might elucidate the possible role of *TLR1A* in APEC resistance.
However, it is unlikely that the observed differences in APEC resistance are solely the direct result of *TLR1A* variants: in addition to TLR expression differences, the Lamont-group also found evidence for differences in adaptive immunity between APEC resistant/susceptible chickens (Sandford et al., 2011; Sandford et al., 2012; Sun et al., 2015b, a; Sun et al., 2016a; Sun et al., 2016b). But this might be a result of the described expression differences in innate immunity. Nevertheless, unpublished results of Matthijs et al. (in preparation) showed that a high(er) APEC resistant broiler line had on average higher levels of KLH-binding NAb compared to a low(er) APEC resistant broilers. In addition, *N*-glycans present on KLH were shown to have structural similarity to *E. coli N*-glycans (Sundsmo et al., 2012), providing a possible direct link between KLH-binding NAb, and APEC resistance.
Breeding for increased resistance, i.e. "the ability of the host animal to exert control over the parasite or pathogen lifecycle" (Bishop, 2012), has the potential risk of co-evolution of pathogens for increased pathogenicity (Woolhouse et al., 2002; Kause, 2011; Thrall et al., 2012). Yet few studies have linked adaptation of the pathogen to increased host resistance (e.g. Decaestecker et al. (2007)). Certainly, co-evolution is a potential risk, which should be monitored closely, if NAb selection is applied on a larger scale. However, (opportunistic) bacteria might be less under selective pressure by NAb selection. Opportunistic bacteria are part of the normal environmental microbiota of an individual, and infect only when resistance is (temporarily) reduced. Using a loose definition, all bacteria can be considered opportunistic pathogens. Preventing bacterial infections might be achieved by controlling bacteria in their normal habitat (e.g. gastrointestinal tract). In case of infection, extracellular bacterial infections are mainly controlled through humoral immunity. NAb, as part of the humoral immunity, might be important controlling effectors of bacteria at the normal habitat, and in case of infection. In addition, NAb likely recognize a broad range of pathogenic epitopes, which makes immune evasion for pathogens more difficult in contrast to antibiotics, and specific vaccinations (Gandon et al., 2001; Mackinnon et al., 2004; Van Boven et al., 2005). Therefore selection for increased non-specific NAb might reduce the number of (bacterial) infections, without increasing specific selective pressure on the pathogenicity of bacteria, and possibly also of other pathogen types.

Concluding remarks

Testing for (increased) general disease resistance by selective breeding for NAb levels is limited: it is not possible to test for a *general* effect. Therefore, an infection with a

gram-negative bacterial pathogen was chosen as a proof-of-principle (tested in two separate experiments). The obtained results of NAb selection for resistance for one gram-negative bacteria at a certain age, and administered via a certain route are no guarantee for similar results at other ages, via other routes, or resistance for other gram-negative bacteria, let alone resistance for gram-positive bacteria, viruses, fungi, and parasites. However, *N*-glycans present on KLH were not only shown to have structural similarity to *E. coli N*-glycans, but also to gram-positive bacteria, viruses, fungi, and parasites (Sundsmo et al., 2012). This gives possibilities of increased general disease resistance as a result of increased KLH-binding NAb levels.

Also, TLR expression, and TLR(1) signaling have been reported to change with age (in mammals) (Jing et al., 2009; Panda et al., 2010; Kollmann et al., 2012), which suggests that disease resistance as a result of the *TLR1A* variants might differ with age as well. This should be investigated in future studies.

One way to obtain a better understanding of general disease resistance, is to investigate 'normal' mortality in the NAb selection lines. Although not statistically tested, no clear differences in mortality were seen. This could be a result of a lack of pathogenic pressure in the environment (i.e. research facility "Carus" of Wageningen University & Research) of the NAb selection lines. Another way to obtain a better understanding is to test the NAb selection lines under commercial settings. Currently, the project group is performing a recurrent field test of crossbred offspring of the High line, and Low line (in collaboration with Hendrix Genetics). Mortality, and the moment, and cause of mortality in life will be an important indicator of possible increased general disease resistance by high(er) NAb levels.

Immunological correlated responses

A potential consequence of selection for any immune trait, or specific disease resistance is that selective breeding has negative consequences on other immune traits, which may result in increased susceptibility for other diseases (Stear et al., 2001). A clear example is the humoral adaptive immunity versus cell-mediated adaptive immunity: increase of humoral adaptive immunity may result in an increase in antibody-dependent disease resistance (e.g. bacterial disease resistance). However, this might result in reduced cell-mediated adaptive immunity, and reduced cell-mediated-dependent disease resistance (e.g. viral disease resistance) (Stear et al., 2001). Indeed, several selection experiments showed (at least some) negative correlated responses (e.g. Gross et al. (1980); Kreukniet et al. (1994); Parmentier et al. (1995)). Others did not observe negative correlated responses (e.g. Li et al. (2008); Pinard-Van der Laan (2002); Sarker et al. (2000)). Although the observation of correlated responses is dependent on the selection criterion (e.g. age measured, type of stimulus, time after stimulus). In addition, observed differences do not necessarily reflect differences in disease resistance, but might reflect (a shift to) a different immune strategy.

To investigate some possible immunological correlated responses of NAb selection, several parameters were measured during the selection experiment: antibodies, immune cell types,

and organ weights. The main correlated responses of the selection criterion were discussed before: KLH-binding IgM, and IgG at 16 weeks of age, and APEC resistance around 2 weeks of age were positively correlated to the selection criterion.

Line differences in antibodies

Age differences

KLH-binding IgT, IgM, and IgG NAb titers were measured at 8 weeks of age, 16 weeks of age, and 32 weeks of age in all females of generation 4 (Table 4). All NAb (iso)types at all three time points were significantly higher in the High line compared to the Low line. However, the difference was smaller at 8 weeks of age compared to 16 weeks of age, and 32 weeks of age. Correlations between the individual's NAb (iso)types at different time points within one line were also estimated (Table 5). In general, NAb (iso)types within a time point correlated moderately. NAb (iso)types between time points correlated low to moderate, and IgM always showed the strongest correlation between time points. These results suggest that NAb levels at 8 weeks of age are not a good (phenotypic) indicator for NAb levels at 32 weeks of age, and are only a moderate indicator for NAb levels at 16 weeks of age. However, NAb levels at 16 weeks of age are a fair (phenotypic) indicator for NAb levels at 32 weeks of age, which is stronger for IgM compared to IgT, and IgG. This is likely the result of environmental stimuli, required for class-switching to IgG NAb levels (Bekeredjian-Ding et al., 2009), affecting the levels of IgG, and IgT. Both the increase in differences of NAb titers over time, and the correlations between the different time points suggest that the line difference of the selection is established before 32 weeks of age. It also suggest that (the majority of) the selection effect has already been established at 16 weeks of age, but not yet at 8 weeks of age. (Around) 16 weeks of age seems therefore a good moment to measure NAb levels as a phenotypic indication for NAb levels during the adult life, but more sampling moments at different ages are required for further details. Genetic analyses (i.e. genetic

Table 4. KLH-binding IgT, IgM, and IgG NAb titers (and SD), and least square line differences (and SE) at 8 weeks of age, 16 weeks of age, and 32 weeks of age in all females of generation 4. General linear models were used to statistically test for line differences: all NAb (iso)type titers at all time points were significantly different between the High line, and the Low line. For the High line 215-233 observations were used, and for the Low line 237-256 observations were used.

		High line	Low line	Difference
8 weeks of age	IgT	6.0 (0.1)	4.7 (0.1)	1.3 (0.1)
	IgM	5.9 (0.1)	4.6 (0.1)	1.3 (0.1)
	IgG	4.4 (0.1)	3.6 (0.1)	0.8 (0.1)
16 weeks of age	IgT	6.5 (0.1)	4.3 (0.1)	2.2 (0.2)
	IgM	6.4 (0.1)	4.6 (0.1)	1.9 (0.1)
	IgG	6.0 (0.1)	4.4 (0.1)	1.6 (0.1)
32 weeks of age	IgT	8.8 (0.1)	6.5 (0.1)	2.4 (0.2)
	IgM	7.7 (0.1)	5.9 (0.1)	1.8 (0.1)
	IgG	7.3 (0.1)	5.3 (0.1)	2.0 (0.2)

correlations) of NAb levels at different ages will provide more information on (dis)similarity of genetic backgrounds of NAb variation at different ages.

The low correlation between NAb levels at 8 weeks of age, and the selection criterion suggests that protective effects of NAb levels at 16 weeks of age might not be the (main) protective effect at younger age. Although the protective effect at such young age can be the result of maternal antibody levels: maternal antibody levels were mostly higher in the High line compared to the Low line (data not shown), but were also found to not be of significant protective influence on APEC survival. Taken together, this implies that APEC resistance observed at 2 weeks of age is possibly not because of selection for higher NAb levels at 16 weeks of age.

The correlations between different NAb types, and at different time points were stronger in the Low line compared to the High line (Table 5). This suggests that the Low line is less sensitive to stimuli that affect NAb levels. In other words, higher NAb levels might be the result of higher sensitivity to stimuli. Also, differences in affinity of KLH-binding IgM, and IgG NAb at 33 weeks of age in generation 2 were observed: the High line had a higher affinity compared to the Low line (chapter 5). Remarkably, no affinity differences were observed for PPD-binding NAb, and HuSA-binding NAb. This suggests that antigen affinity of antibodies might depend on sensitivity to the particular stimuli, possibly by cross-reactivity.

NAb, and NAAb differences

In addition to KLH-binding NAb, several other NAb, and natural auto-antibodies (NAAb) at different ages were measured in the NAb selection lines (Table 6)[4]. In general, IgT, and IgG antibodies, and especially IgM antibodies were always higher in the High line compared to the Low line in different generations between 8 weeks of age, and 63 weeks of age. This suggests that divergent selection for total KLH-binding NAb has a divergent correlated response on a number of (unrelated) other NAb, and natural auto-antibodies (NAAb) at several moments in life, and has possibly a life-long effect on any antibody level.

SpAb differences

Specific antibody (SpAb) responses have been suggested to be dependent on NAb levels before immunization (Lammers et al., 2004; Panda et al., 2015; Cecchini et al., 2016). Also, higher NAb levels were associated with selection for SRBC-binding SpAb levels in chicken (Parmentier et al., 2004a; Cotter et al., 2005; Wijga et al., 2009). HuSA (T helper 2 (Th2) cell-stimulating) SpAb levels were shown to be positively influenced by HuSA-binding NAb, as a result of selection on KLH-binding NAb (chapter 5). This effect was not observed for

[4] I would like to thank Mike. G.B. Nieuwland (Adaptation Physiology, Department of Animal Sciences, Wageningen University & Research), and Mandy Bao (Animal Breeding and Genomics, Department of Animal Sciences, Wageningen University & Research) for measuring the RRBC-binding NAb (Mike), and the NAAb (Mike, and Mandy).

Table 5. Correlations (shown as High line/Low line) between the individual's KLH-binding IgT, IgM, and IgG NAb titers 8 weeks of age, 16 weeks of age, and 32 weeks of age in all females within one line of generation 4. For the High line 215-233 observations were used, and for the Low line 237-256 observations were used. Pearson correlation statistics were used to statistically test if the Pearson correlation coefficients were significantly different from 0. Correlations shown in *italic* were not significantly different from 0. Correlations of a NAb (iso)type between different time points are shown with a grey background.

High/Low		8 weeks of age			16 weeks of age			32 weeks of age		
		IgT	IgM	IgG	IgT	IgM	IgG	IgT	IgM	IgG
8 weeks of age	IgT	-								
	IgM	0.61/0.63	-							
	IgG	0.71/0.68	0.24/0.24	-						
16 weeks of age	IgT	0.21/0.29	0.23/0.32	0.19/0.14	-					
	IgM	*0.09*/0.37	0.22/0.54	*0.01/0.12*	0.55/0.58	-				
	IgG	0.21/0.24	0.18/0.20	0.21/0.18	0.91/0.91	0.40/0.42	-			
32 weeks of age	IgT	0.10/0.28	0.16/0.29	*0.02*/0.19	0.33/0.60	0.29/0.52	0.30/0.55	-		
	IgM	*0.03*/0.29	*0.09*/0.35	*–0.04/0.11*	*0.11*/0.43	0.46/0.62	*0.05*/0.33	0.49/0.71	-	
	IgG	0.09/0.27	0.20/0.27	0.03/0.20	0.38/0.64	0.27/0.46	0.37/0.62	0.77/0.92	0.39/0.55	-

KLH-binding SpAb, and PPD-binding SpAb, even though line differences were present for KLH-binding NAb, and PPD-binding NAb. Possibly this is a result of KLH's, and PPD's Th1 cell-stimulating properties (Sacco et al., 2002; Forbes et al., 2008; Chow et al., 2012), which might have skewed the immune responses to cell-mediated adaptive immunity instead of humoral adaptive immunity. Also no line difference in the dynamics of the SpAb responses, or SpAb affinity were found for any of the used antigens. The results suggest that NAb selection influences the strength of the humoral adaptive immune response against certain antigens (i.e. Th2 cell-stimulating), but not all (i.e. Th1 cell-stimulating).

Moreover, chapter 5 investigated the response of model antigens, without any adjuvant-like components (e.g. lipopolysaccharide (LPS), lipoteichoic acid (LTA)). This could mean that pathogens, and vaccination might overrule the effect of divergent selection on NAb by immune stimulating components, and therefore might result in no differences in SpAb responses. Thus, a protective effect of NAb levels against pathogens might not work through the facilitation of the SpAb response, at least not at the stage of selection described in chapter 5 (selected female chickens of generation 2 at 33 weeks of age).

Total antibody concentration differences

KLH-binding NAb are part of the total amount of antibodies circulating in the blood of an individual. In the GWAS population, IgM NAb was highly

Table 6. Overview of NAb selection line differences for NAb, and natural auto-antibody levels (NAAb) (type) for different (iso)types binding different antigens at multiple ages in several generations.

Type	(Iso)type	Binding[a]	Age	Generation	High vs. Low
NAb	IgT, IgM, IgG	PPD	33 weeks of age	2	>
		HuSA			>
	IgM	APEC lysate[b]	35 weeks of age	3[c]	>
	IgG				=
	IgM		63 weeks of age	5[c]	≥
	IgG				>
	Agglutination (IgT)	RRBC	16 weeks of age	4	>
	IgM, IgG	Pam_3CSK_4[b]	16 weeks of age	6[d]	>
NAAb[b]	IgM, IgG	Ovalbumin	16 weeks of age	4&5	>
		Lysozyme	16 weeks of age	4&5	>

[a] Abbreviations: PPD: avian tuberculin purified protein derivative of *Mycobacterium avium*; HuSA: Human serum albumin; APEC: avian pathogenic *Escherichia coli*; RRBC: rabbit red blood cells; Pam_3CSK_4: Synthetic triacylated lipoprotein (TLR1/TLR2 ligand).

[b] APEC lysate-binding antibodies, and Pam_3CSK_4-binding antibodies, and NAAb might not be truly 'natural', as these antigens (or similar antigen-structures) may be encountered by an individual.

[c] Only selected parents.

[d] Based on 10 randomly selected females per line.

genetically correlated to total IgM antibody concentration, and also *TLR1A* was found to similarly affect total IgM antibody concentration (chapter 4). To investigate correlated selection responses of NAb selection on total antibody concentrations, blood of 30 female chickens of generation 6 at 20 weeks of age was collected, and analyzed for NAb levels, and total antibody concentrations. To create the largest phenotypic differences based on the selection criterion, 5 chickens per *TLR1A* genotype class per line were chosen based on total KLH-binding NAb titers at 16 weeks of age. Samples were analyzed for line, or genotype class differences with a Kruskal-Wallis test. Differences of genotype class within lines could not be estimated due to a low number of samples. Differences of genotype classes, regardless of line, were not found to be significant. Significant line differences were found for KLH-binding NAb titers, and antibody concentrations, except for total IgA antibody concentration (Table 7). This suggests that selection for total KLH-binding NAb at 16 weeks of age increase all KLH-binding antibody types simultaneously. Also, NAb selection increases total IgM antibody concentration, and total IgG antibody concentration (and consequentially total antibody concentration), but not total IgA antibody concentrations. These results, except for total IgM antibody concentration, are not in line with the estimated genetic correlations, and the phenotypic correlations in chapter 4, which predicted opposite findings of the current results. However, the line differences might also be a result of the NAb selection lines (i.e. different populations), which is likely different compared to the GWAS population.

Table 7. Means (and SD) of total KLH-binding NAb titer (IgT), total antibody concentration (tIgT) (µg/mL), and for the isotypes (IgM, IgA, and IgG, and tIgM, tIgA, and tIgG), and differences (High line – Low line) at 20 weeks of age of 15 selected females per line of generation 6. Kruskal-Wallis tests were used to test for significant line differences, which are indicated with different superscript letters within a column ([a], or [b]).

	KLH-binding NAb titer				Antibody concentration (µg/mL)			
	IgT[1]	IgM	IgA	IgG	tIgT[1]	tIgM	tIgA	tIgG
High line	8.9[a] (1.9)	9.3[a] (1.0)	7.1[a] (2.0)	9.3[a] (1.1)	8,098[a] (2,947)	942[a] (152)	273 (126)	6,882[a] (2,979)
Low line	2.7[b] (1.2)	4.6[b] (1.7)	5.2[b] (1.5)	3.4[b] (1.7)	4,365[b] (1,485)	340[b] (132)	252 (81)	3,774[b] (1,446)
Difference	6.1	4.7	1.9	5.9	3,733	602	21	3,108

[1] Chickens were selected on IgT differences at 16 weeks of age.
[2] Summation of tIgM + tIgA + tIgG.

Line differences in immune cell types

To investigate correlated response on peripheral blood mononuclear cells (PBMC), PBMC of the above described 30 female chickens of generation 6 at 20 weeks of age were isolated. Isolated PBMC were stained with monoclonal antibodies specific for chicken T cell subsets (CD4+, CD8+, CD4+CD8+, or γδ), B cells, antigen-presenting cells (APC), natural killer (NK) cells, and thrombocytes, and analyzed by flow cytometry (fluorescence-activated cell sorting; FACS)[5].

The percentage of lymphocytes (based on forward, and side scatter) was associated with *TLR1A* genotype classes ($p = 0.07$; data not shown): on average CC had 75.9% lymphocytes, CG had 83.2% lymphocytes, and GG had 73.0% lymphocytes. Only CG tended to significant difference with GG. This suggests that the percentage of lymphocytes is dependent on *TLR1A*. Because of *TLR1A*'s association with the NAb selection, the increased percentage of lymphocytes could be dependent on NAb selection as well, but no line effect was found. An increased sample size might result in significant line differences.

The percentage of B cells was influenced by NAb selection ($p = 0.07$; Figure 4, left): on average the High line had 4.0% B cells, and the Low line had 1.8% B cells. This implies that NAb levels (and total antibody concentrations) are related to the number of B cells. The underlying assumption is that of a constant fraction of (NAb-producing) B cells is continuously present in the blood.

Also the percentage of thrombocytes was influenced by NAb selection ($p = 0.01$; Figure 4, right): on average the High line had 38.4% thrombocytes, and the Low line had 15.2%. Avian thrombocytes have, in contrast to the mammalian counterpart (i.e. platelets), a role in the immune system (e.g. St. Paul et al. (2012), Ferdous et al.

[5] I would like to thank Christine A. Jansen and Daphne A. van Haarlem (Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University) for their help in designing the experiment, analyzing the samples, processing the results, and valuable discussion.

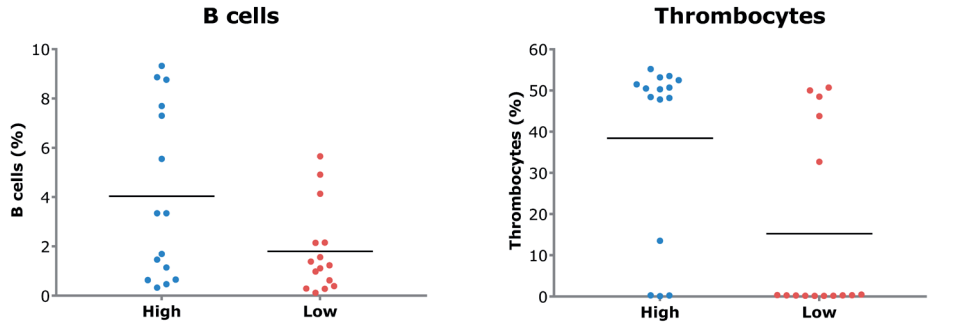


Figure 4. The percentage of peripheral B cells (left), and the percentage of peripheral thrombocytes (right) at 20 weeks of age of 15 selected females per line of generation 6. Kruskal-Wallis tests were used to statistically test for line differences: the percentage of peripheral B cells, and the percentage of peripheral thrombocytes were significantly different between the High line, and the Low line.

(2017)), but the reason for their apparent influence on NAb levels is not clear. Alternatively, as is shown in Figure 4, several individuals had extreme low values (<0.5%), which could be a result of two, or more variants (i.e. alleles) of the thrombocyte marker (CD41/61) present in the population. At least one of these variants might not be recognized by the used monoclonal antibody, due to, for example, small changes in folding of the marker. Future research should confirm if different marker variants are indeed present.

No significant differences in percentages of APC, and NK cells were found between the NAb selection lines, suggesting APC, and NK cells were not affected by NAb selection. Also, no significant differences in percentages of T cell populations were found for line, or genotype, or both, which is an indication that (Th1-dependent) cellular immunity might not be affected as a result of NAb selection. This is in line with the SpAb responses reported in chapter 5, where no line differences in SpAb responses were observed to Th1 cell-stimulating antigens. However, functional test (e.g. phagocytosis capacity, DTH response, cytokine production) are required to test if APC, NK cells, and T cell response are also functionally not affected.

Line differences in organ weights

Organ weights were investigated in 15 day old PBS-inoculated chickens of generation 4, and generation 6 of the APEC infection experiments (inoculated at 8 days of age; chapter 6). Weights of the bursa, spleen, and liver were collected, and corrected for body weight by taking body weight at collection as a covariate into the model.

Already at 15 days of age, the weight of the bursa was significantly heavier in the High line compared to the Low line. The High line's bursa was 13% heavier than the Low line in generation 4, and 26% heavier in generation 6 (data not shown). This suggests that the High line might generate, or might have more capacity to generate naive B cells compared to the Low line. This can result in a higher number of B cells in total, and consequentially a higher (natural) antibody level in the blood.

Similar to the bursa, the spleen at 15 days of age was significantly heavier in the High line compared to the Low line. The High line's spleen was 14% heavier than the Low line in generation 4, and 28% heavier in generation 6 (data not shown). Assuming that the distribution of cell types in the spleen is more or less equal between lines, this could be a consequence of more B cells residing in the spleen.
Liver weight did not differ between lines at 15 days of age. Although the liver has a well-defined function in immunity, like clearance of waste products (homeostasis), production of acute-phase proteins (Juul-Madsen et al., 2014), and the presence of Kupffer cells (Balic et al., 2014), it is not a lymphoid organ, and might therefore not have been affected by NAb selection (at 15 days of age).

Concluding remarks

Possible immunological correlated responses of NAb selection were investigated to identify underlying mechanisms of higher NAb levels (discussed in the next section), and to identify possible detrimental effects. Negative immunological correlated responses were observed in selection experiments for SRBC-binding SpAb levels: resistance to *Escherichia coli*, and *Staphylococcus aureus* infection were lower in the high SpAb line, while resistance to *Mycoplasma gallisepticum*, *Eimeria necatrix*, a splenomeglia virus, and feather mites were higher (Gross et al., 1980). Cellular immunity was found to be lower in the high line in another selection experiment on SRBC-binding SpAb: the high SRBC-binding SpAb line had relatively lower levels of CD8+ T cells, and γδ T cells, but had higher levels of B cells in the spleen, higher complement levels, and a relatively heavier spleen, and bursa weight compared to the low SRBC-binding SpAb line (Kreukniet et al. (1994); Parmentier et al. (1995); Kreukniet et al. (1996); Parmentier, and Van der Poel, personal communication). This SRBC-selection experiment shares similarities with the NAb-selection experiment, although no negative effects of NAb selection on cellular immunity, or other immunological traits were found. However, functional differences cannot be investigated only based on the quantity of a trait, and more research on innate, and adaptive immune activation is required: e.g. phagocytic capacity, antigen presentation, and cellular immunity. Of particular interest could be to investigate responses of different parts of the immune system on APEC.
As mentioned before, line differences in antibodies, immune cell types, and organ weight, and compositions at different ages will provide more information on immunological correlated responses. Especially, because TLR functioning is age-dependent (Jing et al., 2009; Panda et al., 2010; Kollmann et al., 2012). It is also important to point out that *TLR1A* is not only present on B cells, but is also present on several cell types (Iqbal et al., 2005). Although no differences between *TLR1A* variants were detected so-far, it will likely also influence these cell types (in different ways).

The role of natural antibodies in general disease resistance

So far, I have described many different immune(-related) correlated responses of the NAb selection (see Table 8 for an overview). Based on those results, I hypothesize that IgM natural antibodies binding KLH at 16 weeks of age (i.e. adolescent age) reflect the number of naive, resting, IgM-only (i.e. IgA−IgG−) B cells present in an individual, and thereby reflect the humoral adaptive baseline immunity. The baseline immunity is the default (i.e. unchallenged) level of the immune system, and is a proxy for the potential of an individual to respond to pathogens by use of its humoral adaptive immunity. Selection on NAb levels affects the humoral adaptive baseline immunity. I will discuss this in more detail.

KLH-binding IgM

IgM natural antibodies are the most abundant type of NAb found in individuals (Baumgarth et al., 2015; Panda et al., 2015). IgM NAb are also described as the most important isotype in respect to opsonisation, inducing of phagocytosis, and facilitation of the adaptive immune response. IgM is the isotype produced at a low level by naive, resting B cells. Assuming that these B cells produce IgM at a more or less constant rate, IgM is a proxy for the number of naive B cells. Naive B cells require at least two environmental triggers (always BCR antigen, in combination with T helper cells, pathogen recognition receptor (PRR, e.g. TLR) ligands, cytokines, or a combination of these) simultaneously to switch from IgM to IgA, or to IgG (Bekeredjian-Ding et al., 2009). This means that IgA levels, and IgG levels are more

Table 8. Overview of differences in immune(-related) correlated responses (traits) in the NAb selection lines at multiple ages in several generations.

Trait	Binding	Age	Generation	High vs. Low
IgT NAb[a]	KLH	16 weeks of age	1-6	>
IgM NAb				>
IgG NAb				>
TLR1A variants	-	-	6	High: C-variant Low: G-variant
Affinity (IgM/IgG)	KLH	33 weeks of age	2	>
NAb/NAAb (IgT/IgM/IgA/IgG)	Several	8-63 weeks of age	2-6	>
SpAb	KLH/PPD(/Th1?)	~35 weeks of age	2	=
	HuSA(/Th2?)			>
Total/IgM/IgG antibody concentration	All	20 weeks of age	6	>
IgA antibody concentration				=
% B cells	-	20 weeks of age	6	>
% Thrombocytes	-			>
Weight bursa	-	15 days of age	4&6	>
Weight spleen	-			>
Weight liver	-			=

[a] Selection criterion.

dependent on the environment compared to IgM, which might also be of influence on the heritabilities for IgA and IgG compared to IgM. IgA NAb, and IgG NAb are therefore not truly 'natural', as is the case for IgM NAb. Moreover, KLH-binding IgM NAb titers were the strongest predictor of survival of layer chickens compared to KLH-binding IgT NAb, and KLH-binding IgG NAb in the studies of Star et al. (2007a)/Sun et al. (2011), and Wondmeneh et al. (2015).

The studies of Star et al. (2007a)/Sun et al. (2011) also show that NAb levels around 20 weeks of age (adolescence) are better predictors for survival than NAb levels at 40 weeks of age, and 65 weeks of age, regardless of NAb (iso)type. Around adolescence the bursa has delivered most of the B cells, and is involuting. IgM NAb at that moment may therefore reflect the number of B cells that the individual will have for the remainder of its life. It shows therefore also the humoral adaptive potential (i.e. baseline immunity), which plays an important role in the individual's survival. This is supported by the results previously described: NAb levels at 8 weeks of age are weakly correlated with NAb at 16 weeks of age, and 32 weeks of age, but NAb at 16 weeks of age are moderately correlated with 32 weeks of age. Individuals at 8 weeks of age do not show the full potential of the bursa yet, because the bursa will keep producing B cells for the next few weeks. In contrast, at 16 weeks of age the bursa has produced the majority of the B cell population. Individual differences present at adolescence are fairly stable, and therefore remain at 32 weeks of age, and likely for the rest of their lives.

Thus selective breeding for total KLH-binding NAb might result in higher IgM NAb, a higher number of naive B cells, and IgM antibodies, a higher humoral adaptive baseline immunity, and a higher survival during an individual's life.

Environmental sensitivity during B cell ontogeny

The production of B cells in the bursa is strongly influenced by exposure to gut-derived particles (Ekino et al., 1979; Ratcliffe et al., 2014): i.e. an individual can genetically be predisposed to produce high levels of B cells, and NAb, but without proper environmental stimulation this predisposition will not be fully exploited. In our selection experiment, lines were housed mixed within one stable, which likely resulted in a similar exposure to microbiota, and environmental particles over all generations. Although it cannot be excluded that gut-derived particles (i.e. microbiota) differed between lines, it is actually the sensitivity to these gut-derived particles (i.e. DAMP/MAMP, in this case particularly TLR1A ligands) during B cell development, that triggered the increase of B cells, and NAb.

The sensitivity to gut-derived particles during B cell development can work through the B cell receptor. KLH is a large antigen with structural similarities of its *N*-glycans with parasites, viruses, and bacteria, including *E. coli*, and *Salmonella* species (Sundsmo et al., 2012). Because of this diversity of structural similarities (i.e. epitopes) of KLH, selection on KLH-binding NAb represents selection for a relatively wide range of different antigen-binding antibodies (in comparison to selection for a 'simple' antigen, e.g. HuSA).

Indeed, a wide-range different antigen-binding natural antibodies were differed between lines at multiple ages.

The sensitivity to gut-derived particles during B cell development can also (simultaneously) work through pathogen-recognition receptors (PRR). Binding of gut-derived particles (i.e. MAMP) during B cell development likely results in B cell maturation, B cell survival, and B cell proliferation, either directly on the B cell itself, or via supporting cytokine production by the stromal cells in the bursal follicular medullae (Ratcliffe et al., 2014). PRR are highly conserved during evolution, and population-wide genetic variation of PRR with a large impact would be unexpected. Surprisingly, genetic variation in a PRR was found: *TLR1A*. Interestingly, this association was found for IgM (NAb, and total antibody concentrations), which suggests that sensitivity by PRR to gut-derived particles (i.e. MAMP) increases naive, resting IgM-only B cells, regardless of the recognized antigen.

Sensitivity to gut-derived particles plays an important role in stimulating B cell development, i.e. humoral adaptive baseline immunity. However, it is not essential for B cell development, in contrast to the presence of the B cell receptor on the cell surface, as described by Ratcliffe et al. (2014). Therefore, a reduced sensitivity (i.e. presence of *TLR1A* variants) does not have critical consequence on B cell development.

Bursa of Fabricius

In addition to sensitivity for gut-derived particles, selection on NAb levels also increased the relative (and absolute) weight of the bursa at 15 days of age in the High line compared to the Low line. This suggests that at this age the bursa has more, and larger follicles, which will result in a higher B cell development, and more naive B cells being produced.

Involution of the bursa is related to sexual maturation (Ciriaco et al., 2003; Fellah et al., 2014), and therefore the onset of sexual maturation might be of influence on NAb titers as well. In the base population, a positive relation was found between IgM NAb, and eggshell whiteness of the first three eggs laid. In the GWAS population (of the same line as the base population), a relation was found between higher egg production between 17 weeks of age, and 24 weeks of age, and the *TLR1A* variants. Both traits are measured during the adolescent stage of an individual, and might suggest a different 'pace of life'-strategy (Lee, 2006). NAb have been suggested to be higher in avian species with a slower life pace, and aimed at a longer lifespan (Lee et al., 2008; Pap et al., 2015). Chickens, strongly selected on two different life spans, differenced also in immune investment: broilers (fast life pace/short life span) seemed to specialize in strong, short-term humoral responses, and layers (slow life pace/long life span) seemed to specialize in long-term humoral responses in combination with strong cellular responses (Koenen et al., 2002; Simon et al., 2014; Simon, 2016). A slower pace of life-strategy with a later onset of maturation, might result in higher NAb levels.

Differences in bursa weight, and the effective life span of the bursa at different time points remain to be investigated in the NAb selection. Current results suggest that the High line is associated with a heavier bursa, and possibly with a slower sexual maturation-strategy.

Genetic variation underlying humoral adaptive baseline immunity

The relation between IgM natural antibodies, and B cell development has been suggested before. Sławińska et al. (2011a) analyzed KLH-binding NAb, LPS-binding antibodies, and LTA-binding antibodies in layer chickens (partly described in Sławińska et al. (2011b)), and analyzed associated candidate genes *in silico*. Sławińska et al. (2011a) concluded that "[...] candidate genes encode proteins predicted to play a role in (i) proliferation, differentiation and function of B lymphocytes; (ii) TLR signalling pathway, and (iii) MAP signalling cascade" (Sławińska et al., 2011a). In addition, KLH-binding NAb at 20 weeks of age in several layer chicken lines were analyzed with a 1,022 dedicated SNP set. A (weak) association between IgM, and a genomic region containing interleukine-6 (*IL-6*) was found (amongst others), but no other B cell development-related associations were detected (Biscarini et al., 2010; Sun et al., 2013a). In cows, Cordero-Solorzano et al. (in preparation) found an association of LTA-binding antibody titers in milk with a genomic region containing pre-B lymphocyte gene 2 (*VPREB2*), and *VPREB3* (in humans: V-set pre-B cell surrogate light chain 3). Also LPS-binding antibodies, peptidoglycan (PGN)-binding antibodies, and KLH-binding NAb in milk showed an association with the *VPREB2*/*VPREB3*-region, but to a lesser extent. These genes are thought to be involved in B cell maturation. In humans, Aguirre-Gamboa et al. (2016) suggested that B cell immunity is largely environmental dependent, except for IgM antibodies, and naive, resting IgM-only B cells (Aguirre-Gamboa et al., 2016). Both traits were associated to the same region on chromosome 7 with candidate genes being *RP4-647J21*, *MYO1G*, and *ZMIZ2*. Although the causal variant could not be identified yet, the authors suggest the "[...]QTL is associated with the abundance of peripheral B cell subsets in human peripheral blood". The authors also suggested naive B cells to "[...] be representative for that part of the B cell response that has innate-like features, such as the production of natural antibodies [...]" (Aguirre-Gamboa et al., 2016). The above studies are in line with the described results in this thesis, support the hypothesis of IgM NAb being a proxy for naive, resting, IgM-only B cells, and might suggest that IgM NAb reflect the humoral adaptive baseline immunity in several animal species.
However, none of these above mentioned studies has associated a TLR (or any PRR) to IgM levels, or naive B cells. Jonsson et al. (2017) did find an association of *TLR1* variants on class-switching to IgG, and to a lesser extent to IgA ('corrected' for IgM levels: a "composite trait" (Jonsson et al., 2017)) in humans (Jonsson et al., 2017). Involvement of TLR in class-switching is a well-described phenomena (Bekeredjian-Ding et al., 2009). Jonsson et al. (2017) found that the *TLR1*-variant, associated with IgA, and IgG concentrations (i.e. class-switching), was also negatively associated with IgM concentrations (Jonsson et al., 2017). This is not in line with our results for KLH-binding NAb, where IgT, IgM, and IgG

were positively associated with the same *TLR1A*-variant (C-variant). Performing GWAS with the described composite traits will give more information on the role of *TLR1A* variants in class-switching. However, it has to be pointed out that TLR involvement in B cell development is differently regulated in humans compared to chickens: most animals are dependent on gut-associated lymphoid tissue (GALT) for their B cell development. In birds, this is mainly the bursa of Fabricius. However, primates (e.g. humans), and rodents, (and possibly swine as well) are not dependent on GALT for B cell development, but instead have a continuous replenishment of B cells from the bone marrow (Parra et al., 2013). In addition, different TLR activation leads to skewing of the hematopoietic stem cells in the bone marrow. Interestingly, TLR1/TLR2 activation inhibited precursor B cells development (Hayashi et al., 2005), and skewed hematopoietic stem cells in myeloid progenitor development instead of lymphoid progenitor development (Nagai et al., 2006; De Luca et al., 2009). The detected association of TLR with IgM NAb levels, and IgM antibody concentrations might therefore only be present in GALT-species.

Other selection experiments

Sarker et al. (1999) divergently selected chickens on IgM, or IgG concentration at 10 weeks of age (Sarker et al., 1999), which might be the selection experiment performed with most similarities compared to the NAb selection experiment. Unfortunately, it seems that this selection was terminated after four generations. In response to *Brucella abortus* immunization, the high IgM, and high IgG lines had higher specific antibody production compared to the low IgM, and the low IgG lines (Sarker et al., 2000). No differences in cellular immunity were observed between the selection lines (Sarker et al., 2000). The low IgG line did have enhanced phagocytic ability compared to the high IgG line, but no difference between the IgM lines was observed (Sarker et al., 2000). Others investigated selection of bursa size at hatch in broiler chickens (Temple et al., 1961), but this selection did not affect growth, antibody titers, or mortality after *Salmonella typhimurium* infection (Jaffe et al., 1966). Glick et al. (1967) divergently selected chickens on bursa size at hatch as well: selection did affect antibody response to SRBC, but no other consistent differences were found in total white blood cell counts, lymphocytes (percentage, and amount), neutrophils (percentage, and amount), body weight, and organ sizes (Glick et al., 1967; Yamamoto et al., 1982). Both selection for total IgM antibody concentration, and selection for bursa size investigate correlated responses observed in the NAb selection. However, comparison between the selection experiments is difficult: different traits were analyzed, and moreover, different ages of the selection criterion were taken in different genetic material (i.e. genetic lines).

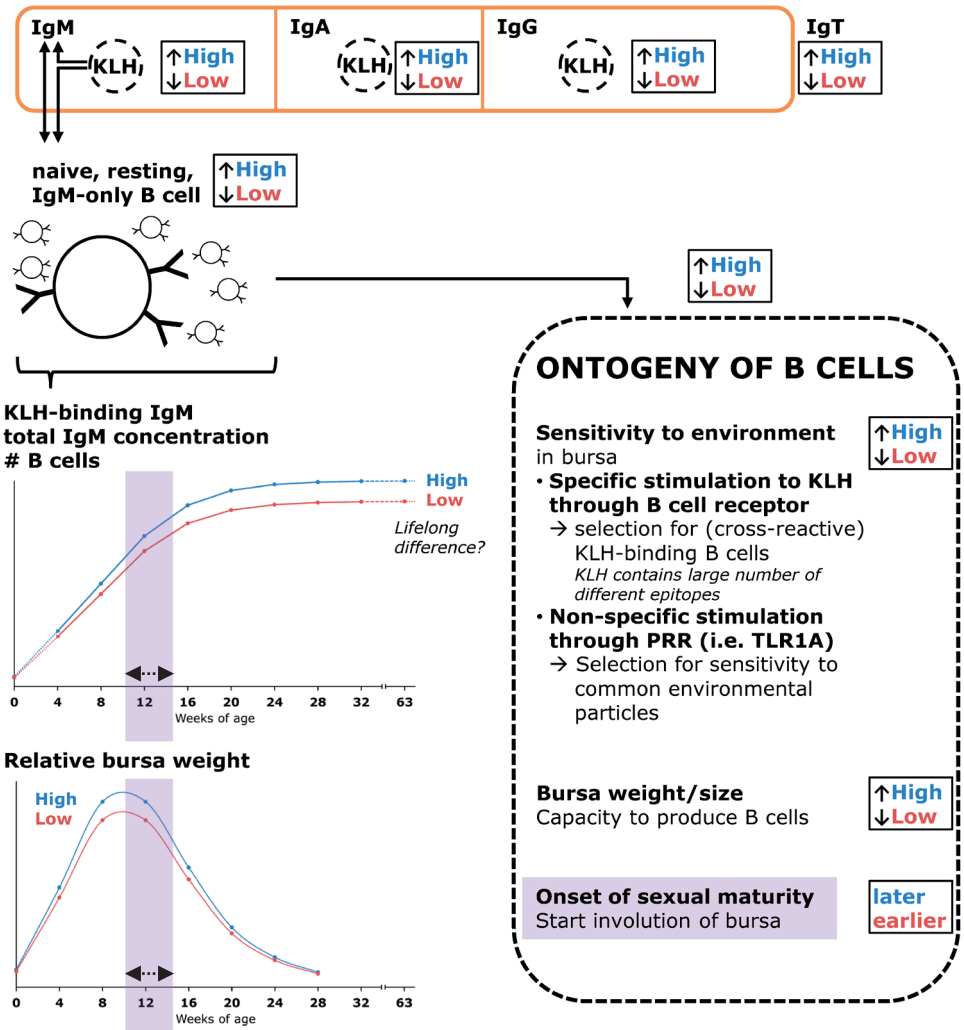
Divergent selection for SRBC-binding SpAb levels shared some similarities with the NAb selection (Pinard et al., 1992). The high SRBC-binding SpAb line had higher levels of B cells in the spleen, and had a relatively heavier spleen, and bursa weight compared to the low SRBC-binding SpAb line (Kreukniet et al. (1994); Parmentier et al. (1995); Kreukniet et al.

(1996); Parmentier, and Van der Poel, personal communication). The SRBC selection might have resulted in higher number of B cells, because of a larger bursa. In addition, an increase of spleen weight might indicate facilitation of antigen-responses, and therefore higher (SRBC-binding) SpAb levels. Although cellular immunity was negatively affected by selection for humoral immunity in these lines, similar to the original Biozzi-model in mice (Biozzi et al., 1970). Nevertheless, at 15 days of age the High NAb line had a heavier spleen compared to the Low NAb line, as seen for the high SRBC-binding SpAb line compared to the low SRBC-binding SpAb line. This could also be a result of an increased number of (naive) B cells residing in the spleen, which might facilitate (IgG) antigen-responses, or both. Histochemistry of the spleen, or FACS of splenocytes at several time moments during immune development, or during an immune response might provide answers to the increased spleen weight.

Concluding remarks

To summarize: I hypothesized that IgM natural antibodies binding KLH at 16 weeks of age (i.e. adolescent age) reflect the number of naive, resting, IgM-only (i.e. IgA−IgG−) B cells present in an individual, and thereby are a proxy for the humoral adaptive baseline immunity (Figure 5). Selection on NAb levels reflects the humoral adaptive baseline immunity, mainly via selection for IgM NAb. IgM NAb represent the number of naive B cells in an individual, i.e. altered ontogeny/proliferation of B cells through 1) altered sensitivity possibly to, or due to a diverse set of gut-derived antigens, and 2) an altered bursa weight, and functional lifespan. NAb selection might also affect antigen-presentation in the spleen, but this is probably not reflected by IgM NAb.

In this PhD study, I did not investigate the identity, and possible localization of specialized NAb-producing B cells (i.e. B1 B cells in humans, and mice). I did neither find (circumstantial) evidence that these specialized NAb-producing B cells exist in chickens, nor find evidence that these B cells do not exist in chickens. This is in line with one of the current ideas that the NAb-producing B cells are too diverse to characterize (Tumas-Brundage et al., 2001; Avrameas, 2016). Actually I hypothesize that NAb producing B cells might not exist at all (in chicken). IgM in general has similar properties as described for IgM NAb: low/moderate affinity, immediate, but limited protection against invading pathogens, and agglutination of bacteria (Pone et al., 2012). I suggest that described NAb, and NAb-producing B cell results in literature are a result of presence of 'normal' antibodies, and B cells in the absence of immune activation, rather than specialized NAb-producing B cells. Of course, certain subpopulations of (N)Ab (for example certain (N)AAb), or subpopulations of B cells might have more important roles in improved general disease resistance, or improved health at certain locations, or difference in development (short lived B cells, long lived B cells, or spleen-originating B cells (Paramithiotis et al., 1993; Ratcliffe et al., 2014)). But the general mechanism behind improved immunity is the development of humoral adaptive baseline immunity. However, simultaneous enhancement of NAb-producing



Author's impression

Figure 5. Hypothesized correlated selection responses of divergent selective breeding on total natural antibody (IgT) levels binding keyhole limpet hemocyanin (KLH) around 16 weeks of age on B cell ontogeny. Figure top shows the genetic potential for antibody levels with KLH-binding antibodies represented by the dashed circle. The correlated selection response of KLH-binding IgM is the hypothesized protective effect on general disease resistance. KLH-binding IgM is a proxy for total IgM concentration, and the number of naive, resting, IgM-only B cells. This suggests that selective breeding on KLH-binding IgM natural antibodies (in this case as a result of selective breeding on total KLH-binding natural antibodies) influences the ontogeny of B cells through 1) changed sensitivity to environment (KLH-specific, and non-specific stimuli), and 2) a changed bursa weight and functional lifespan. In blue the direction of the selection response of the High line, and in red (with arrow) the direction of the selection response of the Low line.

Keep in mind that only part of the proposed relations, and proposed correlated response are supported with experimental data in this thesis.

B cells, and other B cells cannot be excluded based on the presented data. Future research should focus on the detection of subpopulations of antibodies, and B cells with (more) important roles in health of individuals. In addition, baseline immunity of other components of the immune system might provide new direction for increased general disease resistance as well.

Implementation of breeding for general disease resistance

Immunology-related traits in order to improve general disease resistance, have not been implemented in current commercial breeding programs. One reason could be that the study populations used in selection experiments were not directly relevant for breeding companies. This is not the case for the purebred base population, which was chosen to be able to directly implement findings into the breeding scheme. Another possible reason for not implementing could be that selection for improved immunity had undesirable correlated responses on production traits. In this section, possible correlated responses of NAb selection on several production traits, sexual maturation, feather pecking, and non-immunological roles of TLR will be discussed. Also a general recommendation considering current breeding strategies will be given.

Production-related correlated responses

Production traits in the unselected base population

To predict possible negative effects of NAb selection, genetic, and phenotypic relations between KLH-binding NAb titers around 16 weeks of age, and several production traits were investigated (chapter 3). In general, NAb levels had negative, but weak genetic relations with production, but in some cases positive relations were found as well ($r < |0.15|$). However, only few genetic correlations were actually found to be significantly different from 0. A positive genetic correlation ($r = 0.33$) between IgG (and IgA, and IgT) NAb titers, and feed conversion ratio (FCR; consumed feed/egg mass produced) was found. This implies that immune responsive chickens require more energy for an equal egg weight production. Also a negative genetic correlation ($r = -0.29$) between IgG, and eggshell breaking strength was found. These results suggest that small negative correlated responses on production traits might occur as a result of total KLH-binding NAb selection. Or the other way around, in case of selection for total KLH-binding NAb titers around adolescence, a small decrease in production might be expected. However, because of the low genetic correlations, sufficient possibilities are present for simultaneous improvement of both NAb levels, and production traits.

In addition to the estimated correlations, maternal early eggshell whiteness, and eggshell breaking strength affected offspring's IgM NAb (chapter 2). Early eggshell whiteness was positively related to IgM levels, while eggshell breaking strength was negatively related to IgM levels. There is currently no explanation for the observed relation between these

production traits, and NAb levels. It might suggest that the process of eggshell formation (e.g. time spend in oviduct), which could mean that the amount of immunomodulatory substances in the egg might be different, is an important factor influencing the maternal effect.

Body weight in the NAb selection lines

The only production-related trait frequently monitored in all generations of the NAb selection lines is body weight. Body weight was monitored approximately every four weeks for all generations, which allowed for genetic analysis of body weight in the selection experiment[6]. Phenotypic correlations between NAb selection, and body weight were between −0.01-0.05, and were not significant. Genetic correlations between KLH-binding IgT, IgM, and IgG NAb titers with body weight at different ages were between 0.10-0.20 (minimum: $r = 0.03$, maximum $r = 0.23$), though most of them were not significant. It suggests that a small increase in body weight/growth was related to selection for higher NAb levels. Interestingly, the *TLR1A* region was also associated to growth/body weight at several ages (Carlborg et al., 2004; Zhou et al., 2006a; Nadaf et al., 2009; Ankra-Badu et al., 2010; Podisi et al., 2011; Goraga et al., 2012; Podisi et al., 2013; Nassar et al., 2015). This could mean that *TLR1A* is either in linkage disequilibrium with a gene affecting growth/body weight, or that *TLR1A* has a pleiotropic effect on both NAb levels, and growth/body weight. Remarkably, the NAb selection lines differed in body weight from hatch to adult life, but the difference (almost) disappeared when chickens reached peak production (around 35 weeks of age). This means that the NAb selection lines have different growth curves. The High line grows compared to the Low line relatively faster until sexual maturation, and then grows relatively slower until adult body weight. Analyses of weight gain (i.e. growth) between weighing moments in relation to NAb levels will provide more information on the speed of growth between lines, and in relation to the level of NAb.

Literature generally reports significant lower body weights with selection for increased SpAb (against several antigens) immunity, although it has to be realized that such effects dependent on the number of generations, and the selection criterion (immune trait, and age), and might have different effects (e.g. Afraz et al. (1994); Parmentier et al. (1998); Boa-Amponsem et al. (1999); Yunis et al. (2002); Zhao et al. (2012); but see Van der Most et al. (2011) for an overview). These selection experiments also focused on the immune responsiveness of individuals, in contrast to NAb selection. A possibility is that body weight was affected because a standard dose size (in mL) was applied for each chicken. This resulted in a higher dose per kg of body weight in smaller chickens with consequentially a higher immune response compared to larger chickens. However, selection experiments for body weight

[6] These results are based on the MSc thesis of Francois Karangali (Wageningen University & Research), supervised by Mandy Bao, and Henk Bovenhuis (Animal Breeding and Genomics, Department of Animal Sciences, Wageningen University & Research).

resulted in increased mortality for heavy lines compared to lighter lines, and in some cases also decreased antibody production (see Rauw et al. (1998), Rauw (2012), and Van der Most et al. (2011) for an overview). This suggests that body weight changes are not solely the result of the relatively dose/kg body weight applied, but that it also depends on the selection criterion: NAb selection is not the same as SpAb selection.

Production traits in the NAb selection lines

Approximately 40 female chickens per line of generation 5 were monitored for production traits during part of peak production (35-38 weeks of age) (Table 9)[7]. For most production traits, the High line performed equal to the Low line. For some traits, the High line performed significantly higher, although many traits also have a desired optimum. In addition, significant changes were still small, and might not be of biological relevance. The higher egg weight in the High line is likely related to the higher body weight at the moment of lay. Eggshell thickness, and eggshell breaking strength were not significantly different between the selection lines. Even though, a negative relation between NAb levels, and maternal eggshell breaking strength was found in the unselected base population (chapter 2). Average number of eggs laid was not different between lines. FCR was genetically correlated to IgG levels, but was phenotypically not different between the selection lines. The line difference in eggshell whiteness was small, but in line with previously observed correlations: the High line had (slightly) whiter eggs compared to the Low line. It has to be pointed out that the eggshell whiteness was measured around 37 weeks of age (here), and not on the first three eggs laid as previously found (chapter 3). Literature on correlated responses of production traits on antibody-related selection are sparse. No effects of selection for antibody responsiveness were found on egg production (Zhao et al., 2012), and egg weight (Albrecht et al., 2012a). Although, given reported differences in body weight in those studies,

Table 9. Means (and SD) of production traits during part of peak production (35-38 weeks of age; 20 days in total) of selected female chickens per line of generation 5. General linear models were used to statistically correct for the location of the individual in the stable, and to test for line differences, which are indicated with different superscript letters within a row ([a], or [b]).

	High line		Control line		Low line	
n	43		37		41	
Body weight at 37 weeks of age (kg)	1.42[a]	(0.2)	1.38[b]	(0.1)	1.31[b]	(0.1)
Average number of eggs laid	17.9	(2.0)	18.0	(2.1)	17.9	(2.7)
Average egg weight (g)	55.9[a]	(5.6)	53.7[b]	(3.5)	53.2[b]	(4.6)
Average eggshell thickness (mm)	0.45[b]	(0.03)	0.48[a]	(0.03)	0.46[b]	(0.04)
Average eggshell breaking strength (kg)	3.5	(0.4)	3.6	(0.4)	3.7	(0.5)
FCR	2.10	(0.29)	2.10	(0.18)	2.11	(0.29)
Average eggshell whiteness	90.55[a]	(0.81)	90.57[b]	(0.89)	90.51[a]	(1.05)

[7] These results are based on the BSc thesis of Jenny-Helena Söllner (Van Hall Larenstein University of Applied Science). Data was collected by Jenny-Helena Söllner, and Marit Meinsma (Wageningen University & Research).

differences in egg weight might have been expected. Thicker and heavier eggshells were reported for a lighter Low SpAb line compared to the heavier High SpAb line (Albrecht et al., 2012a; Albrecht et al., 2012b), which is observed similarly for the NAb selection experiment. It suggests a pleiotropic effect between antibody production, and eggshell formation.

Concluding remarks

The chickens used in the selection experiment have been selected on production traits for many generations. Relaxation of the selection for the production traits, random genetic drift, and the NAb selection might have reduced production traits compared to commercial circumstances. However in general, production traits do not seem to be strongly negatively correlated to NAb selection. This gives room for simultaneous improvement of NAb levels, and production traits by inclusion of both in the multi-trait selection index (Knap, 2009) under commercial conditions, as is currently done for many (production) traits already. However, it has to be kept in mind that the production traits measured in the NAb selection lines are all below 40 weeks of age, while the majority of production has yet to come. Differences in production at later life, which is known to have more variation, remain to be investigated. Currently, a crossbred product of the NAb selection is being tested under commercial field environment. This field test will provide more information of correlated responses of NAb selection on production traits in commercial layer chickens.

Other correlated responses

Sexual maturation

As mentioned before, maternal eggshell formation (eggshell whiteness, and eggshell breaking strength) was related to NAb levels in the offspring. In addition, a suggestive association between *TLR1A*, and egg production between 17 weeks of age, and 24 weeks of age was found. Finally, delayed sexual maturation might result in a longer functionally active bursa: gonadal steroids have been shown to influence bursal involution (Ciriaco et al., 2003; Fellah et al., 2014). A prolonged period with lower levels of gonadal steroids (i.e. delayed sexual maturation) might result in a prolonged active lifespan of the bursa. This implies that sexual maturation influences NAb levels. This could indicate different pace of life-strategies (Lee, 2006): later sexual maturation might result in increased (humoral) immunity (e.g. NAb levels), and increased lifespan (Koenen et al., 2002; Lee et al., 2008; Pap et al., 2015). Therefore, later sexual maturation might be an indirect selection criterion for poultry breeding companies to improve lifespan of poultry. Sexual maturation is typically measured as ‘age at first egg’. In the past, a strong selection emphasis has been directed to production traits, especially the total number of eggs produced. This might have resulted in a decrease of age at first egg, possibly due to earlier sexual maturation: studies between 1955, and 1970 reported the age at first egg to be between 20, and 25 weeks of age (Abplanalp, 1956; Wright et al., 1968; Acharya et al., 1969), while the NAb selection chickens start producing from as

early as 15 weeks of age, and a reasonable part of the population produces frequently before 20 weeks of age. However, our current housing strategy (mixed line housing until approximately 18 weeks of age) does not allow us to record the exact age at first egg. Previous antibody-related selection experiment studies described positive correlated responses between SpAb, and age at first egg: a later age at first egg was observed for chickens selected on increased SpAb response to SRBC compared to chickens selected on a low SpAb response in two independent selection experiments (Albrecht et al., 2012b; Parmentier et al., 2012; Zhao et al., 2012). These studies are in line with the idea of increased (humoral) immunity with later sexual maturity, although an optimum for later sexual maturation probably is present. Selection on NAb levels might result in later sexual maturation, and reduced egg production during the first part of productive life (17 to 24 weeks of age), but this reduction will be compensated by the (hypothesized) longer productive lifespan.

Feather pecking

In laying poultry, severe feather pecking of conspecifics results in feather damage, and mortality (Savory, 1995), and feather pecking is considered as a serious welfare problem. Kjaer et al. (2003) suggested that selection for egg production resulted in increased behavioral problems (Kjaer et al., 2003). Despite a large number of studies, underlying causal factors are still not known (Nicol et al., 2013; Rodenburg et al., 2013). Malbehavior in the NAb selection lines was observed from 2 days of age (including wounds, and cannibalistic behavior), and was particularly present at young age (around 5 weeks of age). Earlier studies in different experiments found evidence that a link is present between the type of antigen used for immunization, and the location of (feather) pecking (Parmentier et al., 2009). Also, a significant genetic correlation between antibody response due to immunization, and feather pecking was found (Buitenhuis et al., 2004). A study in mice suggested that the level of social interaction might be dependent on the level of immunity (especially interferon (IFN)-γ): individuals with a higher immunity might be able to socially interact more, because these individuals can deal with the increased risk of becoming infected (Filiano et al., 2016). These studies suggest that the High NAb line might be more prone to develop feather pecking behavior. In generation 5, the High line showed increased feather damage compared to the Low line (housed separately), which is in line with literature, even though no difference in feather pecking behavior was observed during observations[8]. Also no line difference in stress-related behavior was observed. NAb levels did tend to be correlated to serotonin levels, and corticosterone levels[8]. Taken together, this suggest that a (weak) relation between behavior, and NAb levels might be present.

[8] These results are based on the MSc theses of Hieu Nguyen Ba, and Kikianne Kroeske (Wageningen University & Research), supervised by T. Bas Rodenburg (Behavioural Ecology, Department of Animal Sciences, Wageningen University & Research).

Non-immunological roles of TLR

The major role of TLR described is recognition of conserved structure on pathogens. However, the role of TLR is more diverse than this: TLR (including TLR1) have been found to be involved in mammalian brain cell renewal (Rolls et al., 2007). These brain TLR are influenced by microbiota (Heijtz et al., 2011), aging (Letiembre et al., 2007; Kaul et al., 2012), and presence of (neuro)diseases (Bsibsi et al., 2002). The availability of chickens with different *TLR1A*-variants (regardless of the NAb selection) give interesting opportunities to study the involvement of TLR on chicken behavior, and brain functioning, and to detect possible other roles of TLR.

Recommendation to the breeding industry

Falconer (1952) suggested that "it would [...] generally be recommended that selection should be carried out under the environmental conditions in which the improved breed is destined to live" (Falconer, 1952). The statement of Falconer (1952) referred to testing for genotype-environment (GxE) interaction, which states that certain genotypes selected for a 'good' overall performance in one environment, might have a 'bad' overall performance in another environment.

In addition to testing of individuals in the destined environment for GxE interaction, another important reason for testing individuals in the destined environment became apparent from this PhD study: the presence of the two variants of *TLR1A* (suggested based on the data), with a respectable effect on the immune system. It suggests a mutation in an evolutionary highly conserved receptor (Leulier et al., 2008; Brownlie et al., 2011). However in genetics, it is also often stated to "use it, or lose it", meaning that if a gene has lost its necessity for the individual, the gene is 'allowed' to mutate, and it loses its function ("natural knockouts", Plenge (2017)), and might, eventually, be completely lost in the genome of a population. It is tempting to speculate that, apparently, *TLR1A* might have lost (a part of) its necessity for the individual. Indeed bacterial recognition for gram-positive, and gram-negative bacteria is covered by other TLR. But this is the case in many species. It is more likely that the current environmental conditions of the base population, a maternal purebred 'elite' line, in the breeding nucleus farms do not provide the necessity to use TLR(1A). In other words, the necessity (i.e. natural selection pressure) is insufficiently present in breeding nucleus farms, possibly due to (too?) successful hygienic barriers set at these locations (Bishop, 2004). Mutations can therefore occur in a random 'obsolete' gene, i.e. obsolete for the environmental conditions of breeding nucleus farms.

Continuing speculation in a broader view, the lack of sufficient pressure of natural selection in the breeding nucleus farms will result in less, or no removal of certain (unfavorable) variants. This increases the probability that those variants remain in the population. By chance, some will have a detrimental effect on the protein functioning, resulting in a reduced function, or loss of function. This effect remains unobserved due to insufficient natural selection, and the set-up of current breeding programs. Current breeding programs deliver

crossbreds of purebred lines as a final product, because of heterosis that arises in these crossbreds. The current strategy of testing poultry is aimed at testing of these crossbred individuals under commercial field conditions. In order to detect the recessive detrimental mutations in purebred lines, a similar (or the same) detrimental mutation needs to be present in the other parental purebred line. Given the random character of mutations occurring in place, and time, this is highly unlikely to happen. Based on sequence data of two other purebred lines, presence of the G-variant (low IgM NAb levels) in these lines can be excluded. One of these lines is the paternal line frequently used on the original purebred line of the NAb selection experiment. In combination with full dominance observed, this cross results in individuals with either a CC-genotype, or a CG-genotype for the *TLR1A*-variant. All crossbred birds have equal levels of IgM NAb. GWAS in crossbred populations, for example for survival, will not detect this mutation. Consequently, the genetic variation present in the parental purebred lines remains undetected.

Therefore I recommend the breeding industry to test purebred lines under commercial field conditions to detect 'hidden genetic variation', because these detrimental mutations are a potential risk for the (genetic) health of the purebred lines. The current environmental conditions in the breeding nucleus farms provide insufficient natural selection, and allow possibly detrimental mutations in purebred lines. The environmental conditions could even allow purebred lines to accumulate several of these mutations, and allow these variants to reach high variant frequencies in the purebred population. Also other livestock breeding at locations with high hygienic barriers (e.g. breeding nucleus farms, or AI stations) might benefit from this. Although the economic value of, for example, a boar, or bull might be too high to risk a potential high impact, or possibly fatal disease under field conditions. It also has to be pointed out that with the current breeding programs no problems have occurred hitherto, and these problems are not likely to occur provided that crossbreds are sufficiently monitored. Also, hidden genetic variation might be present only for mutations that have occurred in only one of the parental crosses, and with a high level of dominance for the original variant. Nevertheless, these detrimental mutations are a potential risk for the (genetic) health of future populations, and should be investigated.

Concluding remarks of this thesis

In the General Introduction of this thesis, I stated that "NAb are heritable, and are easy and (relatively) cheap to measure, and therefore KLH-binding NAb in adolescents might be a suitable indicator trait". The objectives of this thesis were:

1) to investigate the genetic variation of KLH-binding NAb levels in adolescent layer chickens;
2) to investigate the potential of KLH-binding NAb levels as an indicator trait for general disease resistance by
   a) divergently selective breeding on total KLH-binding NAb titers, and
   b) inoculating these NAb selection lines with avian pathogenic *Escherichia coli* (APEC); and
3) to investigate possible correlated selection responses on the immune system, and on production traits.

To summarize my findings:

1) KLH-binding NAb levels around adolescence are heritable. A major effect of *TLR1A* was found to be of influence on KLH-binding IgM NAb;
2) a) Divergent selective breeding on KLH-binding NAb levels is possible, and affects NAb levels at early, and old(er) ages, and has therefore a possible life-long effect,
   b) Selective breeding on higher KLH-binding NAb levels increases APEC resistance (both mortality, and morbidity) at young age in two independent experiments; and
3) Divergent selective breeding on KLH-binding NAb levels results in positively correlated immunological responses (e.g. other NAb at multiple ages, total antibody concentrations, percentages of B cells in the blood), and only few small, negatively correlated production responses might be present (e.g. FCR, eggshell breaking strength).

Several questions remain to be answered, but at this moment the results lead me to the conclusion that: selection on KLH-binding NAb around adolescence increases general bacterial disease resistance, and possibly general disease resistance, and KLH-binding NAb around adolescence can be included in the selection index to improve general disease resistance (as part of the breeding goal). Selection for higher KLH-binding NAb levels can be done by mass selection, or marker-assisted selection. I do recommend a careful approach for selection on NAb levels, because this thesis investigated only one purebred line for only certain correlated responses. Future (field) experiments of crossbred (and purebred?) individuals will provide information on selection on NAb levels on other characteristic, and especially on increased general disease resistance. Nevertheless, the use of a purebred chicken line in our selection experiment, in combination with additional analyses on a blood sample taken as part of normal breeding practices, make the inclusion of KLH-binding NAb around adolescence in the selection index easy to implement.

A question not addressed so far, is if selection on KLH-binding NAb levels should be based on total levels, IgM, IgA, IgG, or a combination of those. Literature, and this thesis suggest that IgM is most representative for general disease resistance, in addition it has the highest heritability. Therefore, I recommend to include KLH-binding IgM NAb titers in the selection index, although all NAb types are expected to increase with IgM due to high genetic correlations.

An important assumption made for this study is that gut-microbiota, and environmental particles did not differently influence NAb levels between the NAb selection lines. This assumptions is probably largely true for sensitivity at MAMP level (i.e. the composition of MAMP in the bursa did not have large difference), and consequential stimulation of TLR in the bursa. However differences of gut-microbiota at specie level were likely present during the whole life of the individuals. The impact of these differences is an interesting area for further study, especially because KLH shows structural similarities to microbiota (Sundsmo et al., 2012), and because of the role of TLR in gut immunity (Cerf-Bensussan et al., 2010; De Kivit et al., 2014). Gut-immunity (or mucosal immunity) is different from peripheral immunity, but might be equally (or even more) important in preventing diseases. Gut immunity of the NAb selection lines (e.g. microbiota composition, gut (immunity) development), and the role of (KLH-binding) IgA in the blood might provide relevant information on improvement of gut immunity.

(Selection for) KLH-binding NAb levels likely also influence general disease resistance in other livestock species, and manipulation of NAb levels might be of interest. However, many of the research on NAb levels is currently done on samples collected during very early life, or at an adult stage, often in combination with a stressful situation (e.g. weaning, or pregnancy). This is likely of major influence on the prediction of NAb for general disease resistance, and health of an individual. Certainly, these samples often are practically convenient samples, or were taken at convenient sampling moments, and are therefore also relevant. However, I do recommend more sampling during several stages of an individual's life, especially around adolescent age.

Other concepts to reduce the impact of diseases are investigated by others as well: longevity/survival, infectivity (and susceptibility/resistance), resilience/robustness, and tolerance (Knap, 2005; Doeschl-Wilson et al., 2012; Lipschutz-Powell et al., 2012). It is beyond the scope of this thesis to discuss these concepts as well, but it can be assumed that NAb levels play a role in these as well. Future studies might investigate the role of NAb levels in reducing fluctuations in individual production by lowering the impact of diseases, or increasing recovery after a disease (i.e. resilience, resistance, and tolerance). Or the role of NAb levels on spreading of diseases within a flock, and herd immunity (i.e. infectivity).

I hypothesized that KLH-binding IgM NAb levels around adolescence reflect humoral adaptive baseline immunity: it represents the potential of an individual to respond to future pathogens by the humoral adaptive immune system. Future studies should investigate baseline immunity of several parts of the immune system on a large number of individuals with genetic information, the "phenomics" approach (Houle et al., 2010). Such studies have

been done in pigs (Flori et al., 2011), and humans in combination with functional *in vitro* assays (Aguirre-Gamboa et al., 2016; Li et al., 2016). These type of studies will increase our understanding of genetic, and phenotypic variation underlying the immune system, and will aid in the identification of novel players in different pathways of the immune system.

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See List of references at the end of this thesis.

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